The mdm2 oncogene encodes a 90-kDa protein that can bind to the p53 tumor suppressor protein and negatively regulate its functions in transcription, cell cycle arrest, and apoptosis. The mdm2 gene is frequently amplified in human sarcomas, which may be responsible for the malignant transformations. We present evidence that the mdm2 oncogene is cleaved by an interleukin 1β-converting enzyme-like protease (caspase) during p53-mediated apoptosis. The protease that cleaves mdm2 has a specificity similar to that of CPP32 (caspase-3), and recombinant caspase-3 is able to cleave mdm2 in vitro. The protease cleavage site has been mapped to between residue 361 and 362 of human mdm2. The proteolytic cleavage removes the COOH-terminal RING finger domain of mdm2, resulting in the loss of RNA binding activity. The p53 binding and inhibition functions of mdm2 are not affected by the cleavage. The cleavage site sequence of mdm2 is evolutionarily conserved, suggesting that regulation by caspase cleavage during apoptosis is an important feature of mdm2.

The mdm2 oncogene was first cloned as an amplified gene on a murine double-minute chromosome in the 3T3DM cell line, a spontaneously transformed derivative of BALB/c 3T3 cells (1). The gene encodes a 489-amino acid polypeptide that contains a p53 binding domain, an acidic region, and three putative zinc-binding motifs (one zinc finger and two RING fingers). It was shown that overexpression of the mdm2 gene in NIH 3T3 cells increased the tumorigenic potential of these cells, thus establishing mdm2 as an oncogene (1). The mdm2 gene can immortalize rat embryo fibroblasts and cooperate with the activated ras oncogene to transform these cells (2). The mdm2 gene is amplified or overexpressed in about 40–60% of human osteogenic sarcomas and about 30% of soft tissue sarcomas (3, 4), implicating its role in the development of these malignancies. An important function of mdm2 is to bind to the p53 tumor suppressor protein, inhibiting its ability to act as a transcription factor (5). p53 plays an essential role in the prevention of tumor development in humans. More than 50% of all human cancers have mutations of the p53 gene (6). Humans or mice carrying germline mutations of p53 are at high risk for the development of multiple forms of malignancies at young ages (7–9). p53 is thought to function by activating the expression of a group of growth regulatory genes (10, 11), leading to cell cycle arrest or apoptosis (12–14).

p53 also activates mdm2 expression at the level of transcription (15, 16), suggesting that mdm2 can function as a negative feedback regulator of p53. Mouse embryos with inactivated mdm2 alleles die shortly after implantation. However, mice carrying inactivated mdm2 and p53 are viable (17, 18). This suggests that an important function of mdm2 is negative regulation of p53. In cell culture experiments, mdm2 overexpression abrogates the ability of p53 to induce cell cycle arrest and apoptosis (19, 20). In addition to regulating p53, mdm2 has been shown to bind to the retinoblastoma protein pRB (21), E2F (22), ribosomal protein L5 (23), RNA (24), and regulate the MyoD transcription factor (25). These activities may also be responsible for or contribute to the transforming properties of mdm2.

An important event during apoptosis is the activation of proteases with homology to interleukin 1β-converting enzyme (ICE), which are now named caspases (26). Caspases are cysteine proteases with the unique property of cleaving Asp-Xaa bonds (where Xaa is a small hydrophobic residue) (27). The critical role of caspases in the control of programmed cell death was first revealed by genetic studies of lower organisms. The nematode Caenorhabditis elegans has an ICE homolog Ced3 that is necessary for the normal developmental cell death in this organism (28). More than 10 homologs of ICE have been isolated (26). Overexpression of caspases can induce apoptosis in mammalian cells (29–31). Conversely, inhibitors of an ICE homolog CPP32 (caspase-3) efficiently inhibit the apoptosis of human cells, suggesting that the proteolytic cleavage of proteins by caspase-3 is important for the induction of apoptosis (32).

To date, a small number of cellular proteins have been found to be substrates of caspases. Some of these substrates are poly(ADP-ribose) polymerase (PARP) (33), sterol regulatory element-binding protein (34), lamin A (35), lamin B1 (36), nonerythroid spectrin (37), actin (38), the retinoblastoma protein pRB (39), DNA-dependent protein kinase (40), the Huntington protein (41), D4-GDP dissociation inhibitor (42), and a 70-kDa component of the U1 spliceosome particle (43). It is not known how proteolytic cleavages of these proteins may result in or contrib-
ute to the apoptosis phenotype. Because some of these known substrates are involved in cellular repair, it has been suggested that destruction of proteins in nuclear homeostatic pathways may be an important mechanism of cell death induction (44).

In this report we show that during p53-mediated apoptosis, the mdm2 protein induced by p53 is cleaved by an apoptosis-specific protease. The cleavage inactivates the RNA binding function of mdm2 without affecting p53 binding. Cleavage by the apoptosis protease is an evolutionarily conserved feature of mdm2, and human, mouse, and Xenopus mdm2 proteins can all serve as substrates for the mouse apoptosis protease. The cleavage site sequence is also present in the recently isolated mdm2 homolog mdmx. These results suggest that cleavage by the apoptosis protease is an important feature of mdm2 which may regulate or inactivate its functions during apoptosis.

**MATERIALS AND METHODS**

**Cloning**—A partial cDNA clone of the human PARP protein was obtained by PCR amplification of a HeLa cDNA library using primers PARP-ATG (5'-CCGGATCCGAGGATGGCGGAGTCTTCG-3') and PARP-UAG (5'-CCGGATCTAAGAGATTTTCTGCGAATC-3'). A 1.1-kilobase PCR product was cleaved with BamHI and inserted into the pBluescript vector (Promega). In *in vitro* translation of the plasmid using the TNT-coupled *in vitro* transcription and translation system (Promega) generated a protein product of ~38 kDa. This PARP fragment contains a cleavage site for CPP32 (caspase-3) between residues 216 and 217 (45).

To obtain the mdm2 mutant representing the protease cleavage fragment (amino acids 1–561), plasmid pHDM1A containing a full-length human mdm2 cDNA (46) was amplified using a primer HDM361N (5'-CCGGATCTCCTAATCGAGAAATCAAGAA-3') and a T7 promoter primer in a PCR with the Pfu DNA polymerase. The PCR product was cleaved with BamHI and cloned into pBluescript. To obtain a clone encoding the COOH-terminal cleavage product (amino acids 362–500), a HDM362–491 (46) was amplified with primers HDM362N (5'-CCGGATCCACCATGTGTTAAAAACTGTGTA-3') and a T7 primer. The PCR product was cleaved with BamHI and cloned into pBluescript. The plasmids were sequenced to confirm that there are no PCR-introduced mutations. To construct mammalian cell expression vectors (pCHDM1–361, pCHDM362–491), these two mutants were excised from the pBluescript vector and inserted into the pCMV-neo-Bam vector (47).

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed using a Chameleon double-stranded DNA mutagenesis kit (Stratagene) according to instructions provided by the manufacturer. The primer HDM361A (5'-CTTCTATGTTCTATGATTAAACAATGGA-3') used to change codon 361 of human mdm2 from aspartic acid to alanine (TGA to TAA) was confirmed by DNA sequencing. The primers were derived from the work of V. Marechal et al. (48).

**Protease Cleavage Assay**—The VM10 cells were maintained at 39 °C in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 500 μg/ml G418, and 200 units/ml hygromycin. To obtain apoptotic cells, the cells grown to ~70% confluence were shifted to a 32 °C incubator for 6 h. The plates were scraped, and both floating and attached cells were harvested by centrifugation at 1,000 rpm for 5 min. The cells were washed three times with lysis buffer, boiled in SDS sample buffer, and the immunoprecipitate was fractionated by SDS-PAGE. The fractionated proteins were transferred to a nitrocellulose filter using a semidyed electrophoresing apparatus for 1 h. The filter was blocked for 5 min with phosphate-buffered saline (PBS) containing 5% non-fat dry milk and then incubated for 1 h with a 1:1,000 dilution of a rabbit anti-mdm2 serum in PBS containing 5% non-fat dry milk. The filter was then washed four times (5 min each) with PBS containing 0.1% Tween 20. The bound primary antibody was detected by incubating for 1 h with protein A-agarose (Boehringer Mannheim) diluted in PBS containing 5% non-fat dry milk. The filter was washed four times with PBS containing 0.1% Tween 20 and then developed using the enhanced chemiluminescence method (ECL, Amersham). Both primary and secondary antibody incubations were carried out at room temperature. An immunoprecipitation was necessary because of the low abundance of mdm2. The short transfer and incubation times reduced the renaturing of heavy chain and its binding by protein A-agarose.

**Recombinant Protease Preparation**—A cDNA clone of the human CPP32 protease was kindly provided by Dr. Emad S. Alnemri (30). This cDNA was used as template for a PCR containing primers p17-ATG (5'-CCGGATCCATGTTATGAAATATCAAGAA-3'), pCHDM1–361 and pCHDM362–491, the Pfu DNA polymerase. The PCR product was cleaved with BamHI and cloned into the pCMV-neo-Bam vector (47), resulting in the addition of 6 histidines to the COOH terminus of CPP32. *Escherichia coli* XL1 blue transformed by the recombinant plasmid was induced by 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h during logarithmic growth. The cells were lysed under nondenaturing conditions, the lysate was passed through a Nl-NTA agarose column (Qiagen) and the column was eluted with imidazole as recommended by the manufacturer. A mock purification was also performed using *E. coli* transformed with the pQE12 vector. SDS-PAGE and silver staining of the purified protease indicated that the recombinant protein was present as two bands of 17 and 12 kDa. A single contaminant band (~50 kDa) with intensity similar to that of the 12-kDa subunit was also present in the purified enzyme preparation as well as the mock purification product. The two enzyme bands and the 17-kDa band (mdm2/xenm2) were extensively confirmed by SDS-PAGE and size ligation of the purified protein, and the cellular debris was removed by centrifugation.

**Glutathione-Sepharose 4B beads (Pharmacia)** were washed with NETN buffer (20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40) containing 0.5% nonfat milk. Twenty μl (packed volume) of beads was incubated with 0.5 μl of *E. coli* lysate for 30 min at 4 °C. The beads were then washed with BFI buffer (20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 2 mM EDTA, 0.1% Nonidet P-40, 2 mM dithiothreitol, 0.05% bovine serum albumin, 5% glycerol). 10 μl of *in vitro* translation lysate containing 35S-labeled mdm2 was mixed with an equal volume of cytosol buffer and incubated with 0.02 μg/ml recombinant CPP32 for 1 h at 37 °C. The cleaved mdm2 was then incubated with the loaded glutathione beads in 200 μl of BFI buffer for 1 h at 4 °C with shaking. The beads were washed with 0.5 × SNNTe (2.5% sucrose, 2.5 mM Tris-Cl (pH 7.5), 250 mM NaCl, 2.5 mM EDTA, 1% Nonidet P-40), boiled in SDS sample buffer, and analyzed by SDS-PAGE.

**Transfection and CAT Assay**—U2-OS cells were maintained in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum. In a transient transfection, 2 × 10⁶ cells were seeded into 10-cm dishes for 24 h and transfected with 5 μg of pCOS1-CAT (gift responsive) (pG1-CAT) and pSDS-PAGE, 1 μg of mdm2 plasmid (pCHDM1A for full-length mdm2 (49), pCHDM1–361 and pCHDM362–491 described above) using the calcium phosphate precipitate protocol. The transfected cells were washed and refed 16 h after the addition of DNA and were harvested 48 h after addition of DNA. 100 μl of cytoplasmic extracts was prepared from each 10-cm plate. CAT assays were performed using 50 μl of extract adjusted to identical protein concentra-
trations in a 150-µl reaction containing 0.8 mm acetyl-coenzyme A, 0.2 µCi of [14C]chloromphenicol, and 0.25 µM Tris-Cl (pH 7.5). Conversion of [14C]chloromphenicol to acetylated [14C]chloromphenicol was detected by thin layer chromatography.

RNA Homopolymer Binding—Poly(A)-agarose, poly(G)-agarose, and poly(C)-agarose were obtained from Sigma. Poly(U)-agarose was obtained from Pharmacia. Human mdm2 was synthesized by in vitro translation of plasmid pHDM1A (46) in the presence of [35S]methionine. The translation product was mixed with an equal volume of cytosol buffer and then incubated with purified recombinant CPP32 (0.02 µg/µl) for 1 h at 37 °C. RNA binding was carried out as described previously (47). The RNA-agarose beads were washed with RNA-binding buffer (20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 5 mM MgCl2, 0.1% Nonidet P-40, 50 µM ZnCl2, 2% glycerol, 1 mM dithiothreitol). 30 µl of the translation-cleavage product was incubated with about 20 µg of RNA in a total volume of 250 µl of RNA-binding buffer. The tubes were rotated for 2 h at 4 °C. The RNA-agarose beads were then washed three times with RNA-binding buffer. The beads were boiled in SDS sample buffer, and the bound proteins were detected by SDS-PAGE and autoradiography.

RESULTS

Activation of a Caspase during p53-induced Apoptosis—A murine cell line (VM10) that expresses a CMV promoter-driven mouse c-myc oncogene and a SV40 promoter-driven temperature-sensitive murine p53 mutant (Val135) was described previously (20). The VM10 cells grow normally at 39 °C when the p53 protein is at a mutant conformation. When the cells are incubated at 32 °C, the p53 protein switches to the wild type conformation, and the cells die by apoptosis. To obtain apoptotic cells, VM10 cells that were maintained at 39 °C were transferred to an 32 °C incubator for 6 h. This treatment causes 30–40% of the cells to fragment their DNA and detach from the plate (20). Cytosol was prepared from these apoptotic cells as well as the nonapoptotic VM10 cells cultured at 39 °C and incubated with a radiolabeled PARP fragment (amino acids 1–343) synthesized by in vitro translation. We found that the PARP fragment was cleaved into a 26-kDa product when incubated with the cytosol prepared from apoptotic VM10 cells but not from the nonapoptotic VM10 cells (data not shown). Therefore, the PARP-cleaving protease, probably CPP32 (caspase-3), is activated during p53-mediated apoptosis in the VM10 cells.

Activation of a mdm2-cleaving Protease during Apoptosis—To determine whether the mdm2 oncoprotein is a substrate of apoptotic proteases, in vitro translated human mdm2 protein was incubated with the VM10 apoptotic and nonapoptotic cytosol. This treatment resulted in the cleavage of human mdm2 into two fragments of 60 and 32 kDa by the apoptotic cytosol (Fig. 1). In the same experiment, mouse and Xenopus mdm2 were also cleaved by the apoptotic VM10 cytosol. This mdm2 cleavage activity is absent in the nonapoptotic cytosol. Other in vitro translated proteins, including p53, TATA-binding protein, and cyclin-dependent kinase inhibitors p57, p21, and p16, were not cleaved in this assay (data not shown), suggesting that the apoptotic protease that cleaves mdm2 is highly specific.

To determine whether the endogenous mdm2 in the VM10 cells is also cleaved during apoptosis, lysates were prepared from the VM10 cells cultured at 39 °C or 32 °C and analyzed by Western blot using anti-mdm2 serum. This experiment showed that the endogenous murine mdm2 is cleaved into a ~55-kDa fragment in cells undergoing apoptosis (Fig. 2). A lower molecular mass 73-kDa protein, which is believed to result from the use of an alternative translation start codon at residue 50 (50), is also cleaved, resulting in the appearance of a double band. No cleavage of mdm2 was observed in the parental cell line Val5 (expresses temperature-sensitive p53 but does not overexpress c-myc) which only undergoes growth arrest after p53 activation.

Using a monoclonal antibody 4B11 that reacts with the COOH terminus of mdm2 (46), we were not able to detect the presence of the COOH-terminal fragment in the apoptotic VM10 cells. When the human U2-OS osteosarcoma cells were transfected with an expression plasmid encoding the COOH-terminal fragment (pCHDM362–491), its expression in these nonapoptotic cells can be detected readily by 4B11 (data not shown), indicating that the absence of detectable COOH-terminal fragment in apoptotic VM10 cells is not the result of technical problems. It is likely that the COOH-terminal fragment is degraded rapidly once generated in the apoptotic cells.

Activation of the mdm2-cleaving protease is not unique to p53-mediated apoptosis. The human leukemia cell line HL60,
which is devoid of endogenous p53, also activates the mdm2-cleaving protease when induced to undergo apoptosis by treatment with the DNA-damaging drug etoposide (data not shown).

Identification of the Protease Cleavage Site on mdm2—Using a series of in vitro translated mdm2 deletion mutants constructed previously (46), the apoptotic cleavage site on mdm2 was mapped to a region between residues 350 and 397 (Fig. 3, A and B). Within this region of mdm2, there is a stretch of amino acids with the sequence 355-EGF DVPD CKK-364 which resembles the known cleavage sites of the cysteine protease CPP32 (RKGDEVD/GVD of PARP, "" represents the cleavage site) (45). This sequence is highly conserved among human, mouse, and Xenopus mdm2 (Fig. 3C). The DVPD sequence is also found in the recently described mdm2 homolog mdmx at the identical position (355-EGI DVPD CRR-364 of mdmx) (51).

To determine whether the conserved DVPD sequence can function as a cleavage site for the protease, synthetic peptides of this region (Fig. 3C) were tested as competitive inhibitors for the cleavage of mdm2 and PARP. We found that the mdm2 peptide can inhibit the cleavage of both mdm2 and PARP (Fig. 4A), indicating that an enzyme with CPP32-like specificity is responsible for the cleavage of both mdm2 and PARP in the VM10 cytosol. Based on the characteristics of the caspases, the carboxyl aspartic residue of the DVPD sequence is expected to be essential for the cleavage. Indeed, a mutant mdm2 peptide that changed the DVPD sequence into DVPA did not inhibit the cleavage of mdm2 or PARP (Fig. 4A). Therefore, the DVPD sequence of mdm2 has properties of a CPP32 cleavage site.

To obtain further evidence that residues 361 and 362 represent the cleavage site in the natural mdm2 protein, the aspartic acid residue at codon 361 was mutated to alanine by site-
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Directed mutagenesis (from DVPD to DVPA). This point mutant was found to be resistant to cleavage by the apoptotic VM10 cytosol (Fig. 4B), indicating that residue 361 is the critical aspartic acid for a caspase. This result also eliminates the possibility that this sequence is merely a buried segment of mdm2 which fortuitously contains the cleavage sequence. These results indicate that the cleavage site on human mdm2 is between residues 361 and 362.

The mdm2-cleaving Protease Has Specificity Similar to CPP32—The cleavage site sequence of mdm2 suggests that the enzyme responsible for the cleavage may be CPP32. To determine whether active CPP32 or an enzyme with similar specificity is present in the VM10 apoptotic cytosol, two specific inhibitors were employed. The tetrapeptide-aldehyde Ac-DEVD-CHO selectively inhibits the CPP32 protease, whereas Ac-YVAD-CHO is selective against ICE (32). When mdm2 was incubated with the VM10 apoptotic cytosol in the presence of those two inhibitors, only Ac-DEVD-CHO inhibited the cleavage of mdm2 at submicromolar concentrations. The ICE-specific inhibitor Ac-YVAD-CHO was completely ineffective (Fig. 5A). This result indicates that CPP32 or an enzyme with similar specificity is present in the VM10 apoptotic cytosol and is responsible for the cleavage of mdm2.

To determine whether mdm2 can be cleaved directly by the CPP32 protease, in vitro translated mdm2 was tested for cleavage by recombinant CPP32. The human CPP32 enzyme was expressed in E. coli as a COOH-terminal histidine-tagged protein. The enzyme was purified by nickel-chelating chromatography and incubated with in vitro translated human mdm2. The recombinant CPP32 enzyme and the VM10 apoptotic cytosol cleaved mdm2 into products that are identical on SDS-PAGE (Fig. 5B). This result indicates that mdm2 is a substrate of CPP32 in vitro, CPP32-cleaved mdm2 at the same site as the apoptotic VM10 cytosol.

The Cleaved mdm2 Still Has the Potential to Inhibit p53—Mapping of the protease cleavage site indicates that mdm2 can be cleaved into two fragments during apoptosis. The NH2-terminal fragment (amino acids 1–361) contains the p53 binding domain, acidic region, and one putative zinc finger motif. The COOH-terminal fragment (amino acids 362–491) contains two highly conserved RING finger motifs (52). Because an important function of mdm2 is to bind to and inhibit p53, we tested whether the mdm2 cleavage fragments are still capable of binding and inhibiting p53.

To determine the p53 binding potential of the cleaved mdm2, in vitro translated mdm2 was incubated with purified recombinant CPP32. The cleavage product was then incubated with glutathione-agarose beads loaded with GST-p53 fusion proteins. Full-length mdm2 binds to the GST-p53 fusion protein specifically in this assay (46). The NH2-terminal fragment of the cleaved mdm2 bound to GST-p53 with efficiency similar to that of the full-length mdm2 (Fig. 6A). The COOH-terminal fragment did not bind to p53. This result indicates that the NH2-terminal fragment of mdm2 is still functional in p53 binding.

To determine the ability of the cleavage product to inhibit p53 transcription function, recombinant plasmids were constructed which express residues 1–361 and 362–491 of human mdm2. A human osteosarcoma cell line U2-OS was transfected with a p53-responsive reporter plasmid pCOSX1-CAT. This plasmid contains p53 binding sites, a basal promoter, and the p53-responsive reporter gene (15). The U2-OS cells contain genotypically wild type p53 (53). When the p53-responsive plasmid pCOSX1-CAT was introduced, a strong expression of CAT was observed compared with the parental plasmid (pTi-CAT), which has no p53 binding site. Cotransfection of a plasmid expressing full-length mdm2 resulted in strong inhibition of CAT expression, indicating that the endogenous p53 in this cell line is functional and active (Fig. 6B).

When residues 1–361 of mdm2 representing the NH2-terminal cleavage fragment were cotransfected with pCOSX1-CAT, a strong inhibition of CAT expression was also observed (Fig. 6B). When the transfected cells were metabolically labeled using [35S]methionine and analyzed by anti-mdm2 immunoprecipitation, the endogenous p53 coprecipitated with the
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The Apoptotic Cleavage Inactivates RNA Binding by mdm2—mdm2 has recently been shown to have properties of an RNA binding protein (24). The major determinant of RNA binding has been mapped to the two COOH-terminal RING fingers. The apoptotic cleavage separates the RING finger region from the p53 binding domain and the acidic region of the protein. This should result in the loss of RNA binding by the large NH2-terminal fragment.

To determine the effect of the cleavage on the RNA binding property of mdm2, in vitro translated mdm2 was incubated with purified recombinant CPP32. The cleavage products were incubated with RNA-agarose beads containing four different homopolymers: poly(A), poly(U), poly(C), and poly(G). It was shown that recombinant mdm2 produced in insect cells binds preferentially to poly(G) and poly(U) (24), a property exhibited by several RNA-binding proteins (54–56). The in vitro translated full-length mdm2 also binds selectively to poly(G) and poly(U) (Fig. 7). When the CPP32-cleaved mdm2 fragments were incubated with the RNA beads, the COOH-terminal fragment showed binding specificity and affinity similar to those of the full-length mdm2. The NH2-terminal fragment only showed very weak binding compared with the COOH-terminal fragment or full-length protein (Fig. 7).

This experiment suggests that the apoptotic cleavage results in the separation of the RNA binding domain of mdm2 from its p53 and L5 binding domains. The large NH2-terminal fragment lost most of its RNA binding activity. The result also indicates that the NH2-terminal fragment cannot bind to RNA by remaining in a complex with the COOH-terminal fragment. In a separate experiment, we immunoprecipitated the cleavage products using monoclonal antibodies against either the NH2-terminal or COOH-terminal fragments of mdm2 and did not observe coprecipitation of the two fragments even at physiological salt concentration (data not shown). These results demonstrate that after the apoptotic cleavage, the two cleavage products do not remain bound to each other.

**DISCUSSION**

Activation of the ICE family of cysteine proteases appears to be a common step in the apoptoses induced by diverse stimuli such as viral infection, DNA damage, and developmental signals. Experiments described above showed that a mouse cell line undergoing p53-induced apoptosis activates a protease that is capable of cleaving the PARP protein, a known substrate of the CPP32 protease. This observation provides further support for the central role of caspase activation during apoptosis induced by diverse stimuli, including p53. A similar observation has also been described in a different cell line system of p53-mediated apoptosis (57).

Apoptosis induction by p53 is, in part, mediated by transcription activation of downstream genes (58–60). p53 also activates the expression of the mdm2 oncogene (15, 16). Induction of mdm2 expression is observed irrespective of the outcome of p53 activation, i.e., growth arrest or apoptosis. Thus, cells destined to undergoing apoptosis after activation of p53 contain high levels of mdm2. Overexpression of mdm2 by transfection can inhibit p53-mediated cell cycle arrest and apoptosis (20). We tested whether the apoptosis-specific proteases can target mdm2, based on the hypothesis that cleavage of mdm2 may eliminate its p53-inhibitory activities, thus promoting the commitment to cell death.

Experiments described in this report demonstrated that the mdm2 protein is a substrate of a caspase during apoptosis. The protease that cleaves mdm2 during apoptosis is likely to be CPP32 (caspase-3) or an enzyme with similar specificity. The protease cleavage site on mdm2 was localized to between residues 361 and 362 of the human protein, distant from the p53 binding domain. Previous deletion mapping studies have suggested that the mdm2 sequences around this cleavage region are not necessary for binding and inhibition of p53 transcription functions (20, 46, 49, 61). As expected, the NH2-terminal cleavage product is still capable of binding p53 and inhibiting its transcription activation function. Therefore, it appears that
the p53-inhibitory functions of mdm2 are not directly affected by this cleavage.

Mdm2 has recently been shown to have RNA binding activity, as well as properties of a nuclear/ cytoplasmic shuttling protein (24). The RNA binding activity has been mapped to the COOH-terminal RING finger motifs, a highly conserved region with no other known function (24). Mdm2 binds specifically to certain RNA homopolymers and to unique synthetic RNA sequences that can form stem-loop structures (24). Furthermore, mdm2 can also bind to the ribosomal L5 protein through its central acidic region (63). L5 is also an RNA-binding protein that can bind to 5 S rRNA (23). These observations suggest that mdm2 may have functions in intracellular trafficking or regulation of other processes through binding to RNA.

The large apoptotic cleavage fragment of mdm2 (residues 1–361) contains the p53 and L5 binding domains. This fragment displayed dramatically reduced ability to bind RNA. The 130-amino acid COOH-terminal fragment retains the RNA binding affinity and specificity of full-length mdm2. This fragment contains no significantly conserved sequences except the two RING fingers, suggesting that its main function is RNA binding. Therefore, one functional consequence of the apoptosis cleavage of mdm2 is inactivation of its RNA binding. This result also suggests that the COOH-terminal fragment represents a relatively independent functional domain. The protease cleavage site may be positioned at a "linker" region. This is also consistent with the observation that the two cleavage fragments do not associate with each other.

The apoptosis proteases may function by destroying important proteins that function in cellular repair or metabolic pathways (44). Alternatively, the cleavages may activate certain substrates that then directly cause cell death. Because mdm2 expression is induced by p53, it may provide p53-inducible cell survival functions during DNA damage response or cellular stress, facilitating cellular repair. These functions may need to be inactivated when cells decide to enter the apoptosis pathway. Alternatively, mdm2 may have cryptic cell death functions that are activated by the protease cleavage and contribute to p53-induced apoptosis. The position of the apoptotic cleavage site on mdm2 suggests that mdm2 functions involving RNA binding may be regulated during apoptosis.

To determine whether the mdm2 cleavage products have a cell death-inducing function, we expressed the mdm2 fragments in U2-OS cells by transfection. These experiments did not reveal obvious cytotoxic effects on normal cells. Using a previously established assay (20), we found that the noncleavable mdm2 point mutant and the NH2-terminal cleavage fragment can inhibit p53-mediated apoptosis in VM10 cells as efficiently as wild type mdm2. Therefore, these limited attempts have not revealed a biological function of the apoptotic

2 J. Roth, M. Dobbelstein, D. A. Freedman, T. Shenk, and A. J. Levine, submitted for publication.

3 J. Chen, unpublished results.
cleavage. It should be emphasized that the assays seeking for mdm2 activity can only measure the effects of mdm2 in nonapoptotic cells. They do not allow us to determine whether the cleavage contributes to events after the activation of the proteases, which is believed to be the commitment step. It is possible that after p53 triggers the activation of the apoptotic proteases, its transcription-related functions are not required for later steps. Therefore, it is not surprising that the cleavage of mdm2 does not affect its p53 transcription-inhibitory function. Several studies have suggested that p53 may have apoptosis-inducing activities distinct from its transcription activation function (59, 62, 64). At present, we do not know whether mdm2 or its apoptotic cleavage product regulates this putative p53 function.

Because it is likely that apoptosis results from a combination of cleavage events, the contribution of individual events may not be detectable by current techniques. Furthermore, the presence of endogenous mdm2 in the cells we used may have prevented us from observing certain effects. It should be noted that at present, we also cannot rule out the possibility that the cleavage of mdm2 is a coincidence unrelated to apoptosis. However, the conservation of the cleavage site sequences in mdm2 of different species and in mdm strongly suggests that this cleavage is an important feature of mdm2.

This report identifies a new target of the ICE family proteases during apoptosis. It suggests a new and direct role of the mdm2 oncoprotein in the apoptosis process and a new interaction between the p53 pathway and the apoptosis pathway, i.e., by proteolytic modification of a p53-inducible protein. The physiological significance of this event remains to be determined. However, the findings described in this report reveal a new aspect of the p53-mdm2 pathway. Further investigation of this phenomenon may shed light on the function of mdm2 and its role in apoptosis.

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REFERENCES
1. Fakhrazadeh, S. S., Trusko, S. P., and George, D. L. (1991) EMBO J. 10, 1565–1569.
2. Finlay, C. A. (1993) Mol. Cell. Biol. 13, 301–306.
3. Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L., and Vogelstein, B. (1993) Nature 363, 80–83.
4. Cordon Cardo, C., Latres, E., Drobnjak, M., Oliva, M. R., Pollack, D., Woodruff, J. M., Marechal, V., Chen, J., Brennan, M. F., and Levine, A. J. (1994) Cancer Res. 54, 794–799.
5. Mamon, J., Ambethi, G. P., Olson, D. C., George, D., and Levine, A. J. (1992) Cell 69, 1237–1245.
6. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991) Nature 348, 705–708.
7. Malkin, D., Li, F. P., Strong, L. C., Fraumeni, J. F., Nelson, C. E., Kim, D. H., Strong, L. C., Fraumeni, J. F., Nelson, C. E., Kim, D. H., Lieber, M. M., and Poirier, G. G. (1990) Cancer Res. 50, 925–930.
8. Srivastava, S., Zou, Z., Pirollo, K., Blattner, W., and Chang, E. H. (1990) EMBO J. 9, 3761–3767.
9. Srivastava, S., Zou, Z., Pirollo, K., Blattner, W., and Chang, E. H. (1990) EMBO J. 9, 3761–3767.
10. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, J., and Levine, A. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9042–9046.
11. Neamaty, N., Fernandez, A., Wright, S., Kiefer, J., and McConkey, D. J. (1995) J. Immunol. 154, 3788–3795.
12. Martinez, J., Georgoff, I., and Levine, A. J. (1991) Nature 351, 337–340.
13. Lin, D., Shields, M. T., Ullrich, S. J., Appella, E., and Mercer, W. E. (1992) EMBO J. 11, 299–305.
14. Martinez, J., Georgoff, I., and Levine, A. J. (1991) Nature 351, 337–340.
15. Wu, X., Bayle, J. H., Olson, D., and Levine, A. J. (1993) Science 259, 5048–5057.
16. Alnemri, E. S., Livingston, D. L., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wang, W., and Yuan, J. (1996) Cell 87, 171–178.
17. Howard, A. D., Kostura, M. J., Thornberry, N. D., Gin, G. J. F., Limjuco, G., Borsuk, B. A., and Jochemsen, A. G. (1996) Mol. Cell. Biol. 16, 5048–5057.
18. Polik, Y., Waldman, T., He, T. C., Kinzler, K. W., and Vogelstein, B. (1996) Cancer Res. 56, 377–382.
19. Chen, X., Ko, L. J., Jayaraman, L., and Prives, C. (1996) Mol. Cell. Biol. 16, 441–454.
20. Chen, J., Xu, W., Lin, J., and Levine, A. J. (1996) Mol. Cell. Biol. 16, 2445–2452.
21. Xiao, X., Chen, J., Levine, A. J., Hodgkiss, P. G., Xiong, J., Sellers, W. R., and Livingston, D. M. (1995) Nature 375, 694–698.
22. Martin, K., Trouche, D., Hagemeier, C., Sorensen, T. S., La Thangue, N. B., and Kouzarides, T. (1995) Nature 375, 691–694.
23. Marechal, V., Elenbaas, B., Piette, J., Nicolas, J., and Levine, A. J. (1994) Mol. Cell. Biol. 14, 7414–7420.
24. Elenbaas, B., Matthias, D., Roth, J., Shenk, T., and Levine, A. J. (1996) Mol. Med. 2, 439–451.
25. Fiddler, T. A., Smith, L., Tapscott, S. J., and Thayer, M. J. (1996) Mol. Cell. Biol. 16, 5048–5057.
26. Marechal, V., Elenbaas, B., Piette, J., Nicolas, J., and Levine, A. J. (1994) Mol. Cell. Biol. 14, 7414–7420.