Identification of the MDM2 Oncoprotein as a Substrate for CPP32-like Apoptotic Proteases*

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Programmed cell death is mediated by members of the interleukin 1-β convertase family of proteases, which are activated in response to diverse cell death stimuli. However, the key substrates of these proteases that are responsible for apoptotic cell death have not been identified. Here we report that the MDM2 oncoprotein is cleaved by members of the CPP32 subfamily of interleukin 1-β convertase proteases both in vitro and in vivo, resulting in the disappearance of MDM2 from apoptotic cells. Because MDM2 functions as a negative regulator of the p53 tumor suppressor and because p53 induces apoptosis in response to a variety of stimuli, this cleavage of MDM2 by CPP32-like proteases may result in deregulation of p53 and contribute directly to the process of apoptotic cell death.

Programmed cell death (apoptosis) plays a key role in normal development as well as in the pathogenesis of many diseases (1, 2). Diverse stimuli utilize cell death effectors that are conserved from nematodes to mammals (3–5). The executioner proteases are the homologous to Ced-3, a protease essential for cell death in Caenorhabditis elegans (6). The mammalian ICE (or caspase) family consists of three subfamilies, designated the ICE-like, the Ichi-like, and the CPP32-like subfamilies (7–9), which show distinct substrate specificities and may play different roles in apoptosis. However, the mechanism by which these proteases induce apoptotic cell death is not known. Substrates of ICE family proteases include nuclear lamins, cytoskeletal components, and proteins involved in DNA repair, RNA processing, and signal transduction (7, 10–12). Cleavage of lamin contributes to fragmentation of apoptotic nuclei (13), but the relevance of other known substrates to cell death is unclear.

Here we report that the oncoprotein MDM2 is a substrate of CPP32-like apoptotic proteases. Because MDM2 is a negative regulator of the tumor suppressor p53, which induces both apoptosis and cell cycle arrest (14–16), these results identify MDM2 as a protease substrate that may play a direct role in the cell death program.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—Wild type mouse mdm2 (17) was a generous gift of Donna L. George (Thomas Jefferson University, Philadelphia, PA). Substitution of aspartate 359 with glutamic acid was accomplished by converting the aspartic acid codon GAA to GGA by Altered Sites II in vitro Mutagenesis System (Promega) using a synthetic oligonucleotide. Both WT and D359E mdm2 were HA-tagged by subcloning into pJ3H (18), then into pcDNA3 (Invitrogen) plasmids.

Transfections— COS-7 cells were transiently transfected with the lipofectamie method as suggested by the manufacturer (Life Technologies, Inc.). Briefly, 15 µl of lipofectamine plus 5 µg of plasmids were incubated with the cells for 5 h, then the medium was replaced with DMEM supplemented with 10% calf serum for 24 h.

Immunoblot Analysis—Cell lysates were electrophoresed and immunoblotted as described (19). All primary antibodies were from Santa Cruz Biotechnology, except for anti-PARP (obtained from G. G. Poirier, Laval University, Quebec, Canada); anti-CPP32 (Transduction Laboratories); and anti-HA (Boehringer Mannheim).

Preparation of Cytosols—Cytosols were prepared essentially as described (20). Briefly, cells were washed twice with phosphate-buffered saline and once with an extraction buffer consisting of 50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl2, 1 mM DTT, 20 mM cytochalasin B, and protease inhibitors. Cells were then resuspended in extraction buffer (100 µl/106 cells), incubated on ice for 20 min, and disrupted with a glass Dounce homogenizer. The cell lysate was centrifuged, first at 10,000 g for 10 min and then at 100,000 g for 90 min at 4 °C. The final supernatant was used as cytosolic extract.

Immuno depletion of Cytosols—Cytosolic extracts were immunoprecipitated twice with either anti-CPP32 or the nonrelated anti-p21 antibody as control. Each immunoprecipitation was carried out with the cells for 5 h, then the medium was replaced with DMEM, incubated for 1 h at 4 °C, and the immunodepleted cytosol was used directly in protease assays.

Preparation of Proteases—(His)6-tagged human CPP32, McH2, Mch3, and mouse ICE were expressed in Escherichia coli (21). Enzymes were either purified by Ni2+ affinity chromatography or the soluble fraction of sonicated bacterial lysates were used directly in protease assays. The activity of enzyme preparations was first determined with synthetic peptide substrates, defining 1 unit of enzyme as the amount needed to release 1 µmole fluorescent aminomethylcoumarine (AMC)/h using 10 µM DEVD-AMC for CPP32, Mch2, and Mch3 and 10 µM YVAD-AMC for ICE.

In Vitro Cleavage Assay—[35S]Labeled MDM2 was prepared by in vitro translation using the TNT-linked transcription/translation kit (Promega, Madison, WI) with T7 polymerase and mouse mdm2 cDNA in a Bluescript KS plasmid. In vitro translated [35S]-MDM2 (2 µl of 50-µl final volume of the TNT reaction) was incubated without proteases or with 5 µg of cytosolic extracts from control or apoptotic cultures or with purified proteases for 1 h at 30 °C in a 20-µl reaction mixture containing 25 mM Hepes, pH 7.5, 0.1% CHAPS, and 1 mM dithiothreitol.

RESULTS AND DISCUSSION

Degradation of MDM2 in Apoptotic Cells—Recent studies have indicated that the phosphatidylinositol (PI) 3-kinase signaling pathway plays a key role in preventing apoptosis of growth factor dependent cells (22, 23). Expression of dominant negative p53 inhibited apoptosis of PC12 and Rat-1 cells in response to either serum deprivation or PI 3-kinase inhibition, suggesting the involvement of p53 in this pathway of apoptotic

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by treatment of cells in growth medium with PI 3-kinase inhibitors, 0.5
or left in growth medium (serum deprivation) for 16 h to induce apoptosis
15050
and apoptosis (22, 23). All cells from control cultures and detached apoptotic cells follow-

mblasts (representative of at least five similar experiments.

A
B
C
D

FIG. 1. Degradation of MDM2 in apoptotic cells. Rat-1 fibro-

FIG. 2. A, cleavage of PARP in apoptotic cells. Cell lysates from Fig.
cells, which provide an efficient source of active apoptotic pro-

FIG. 3. In vitro cleavage of MDM2 by apoptotic cytosol. A, in

and not shown) and in apoptotic PC12 cells after either growth
factor deprivation or treatment with PI 3-kinase inhibitors
(Fig. 1C). In contrast, as in Rat-1 cells, apoptotic PC12 cells
contained increased levels of p53, Bax, p21, and GADD45 pro-

the possible cleavage of MDM2 by apoptotic proteases, we used

an in vitro cleavage assay (20) in which [35S]methionine-la-

b B

C

A

35S-MDM2 was incubated without cytosol or with 5 µg
of cytosolic extracts from control or apoptotic U937 cultures. In lanes 4
and 5, the indicated peptide inhibitors (0.1 µM) were added to the
reactions. MDM2 and its cleavage products were separated on SDS-
polyacrylamide gel and analyzed by a PhosphorImager (Molecular Dy-
namics). The apparent molecular masses of intact MDM2 (90 kDa) and
its fragments (30 and 60 kDa) are marked by arrows. The results are
representative of at least five similar experiments. B, cytosolic
extracts from apoptotic U937 cells were subjected to two sequential immuno-
precipitations with either anti-CPP32 or the nonrelated anti-p21 antibody
as control. In vitro translated 35S-MDM2 was incubated in the presence
of 5 or 10 µg of control (CPP32) and immunodepleted (CPP32)
cytosolic extracts, subjected to polyacrylamide gel electrophoresis, and
analyzed by a PhosphorImager. Data are presented as the amount of
intact MDM2 remaining after indicated times of incubation. The results
are representative of two similar experiments.

2 R. Yao, P. Erhardt, and G. M. Cooper, unpublished observations.
resulted in a loss of intact MDM2 protein, whereas control cytosol from untreated cells had no effect (Fig. 3A, first three lanes). Moreover, with apoptotic cytosol we observed a concomitant appearance of two prominent MDM2 fragments. The sum of the apparent molecular masses of the two fragments (60 and 30 kDa) was approximately equal to the apparent molecular mass of intact MDM2 (90 kDa), indicating that a single site in MDM2 was most sensitive to cleavage by apoptotic proteases.

To identify the apoptotic proteases responsible for cleavage of MDM2, we added ICE protease inhibitors, DEVD-CHO, which potently inhibits both the CPP32-like and the ICE-like subfamilies (26),3 abolished the MDM2 cleavage activity of apoptotic cytosol (Fig. 3A, fifth lane). In contrast, YVAD-CHO, which is a selective inhibitor of the ICE-like subfamily (26), did not affect MDM2 cleavage (Fig. 3A, fourth lane). Thus, MDM2 cleavage in apoptotic cytosol is catalyzed by CPP32-like as opposed to ICE-like proteases.

To further investigate the role of CPP32 in MDM2 cleavage, we immunodepleted apoptotic cytosol with anti-CPP32 antibody. This removed the majority (at least 90%) of CPP32 from the cytosol as assessed by immunoblotting and decreased MDM2 cleavage activity by approximately 50% (Fig. 3B). It thus appears that CPP32 is a major source of MDM2 cleavage in apoptotic cytosol. However, cleavage of MDM2 may also be catalyzed by other members of the CPP32 subfamily.

Cleavage of MDM2 by Purified Proteases—To directly analyze the ability of CPP32 to cleave MDM2, we tested the activity of CPP32 purified from an E. coli expression system, in which CPP32 is activated as a result of autocatalytic cleavage (21). Incubation with purified CPP32 resulted in the initial cleavage of MDM2 to two fragments of the same size as those produced by apoptotic cytosol (Fig. 4A). Incubation with increased amounts of CPP32 (or incubation for longer times; data not shown) resulted in the disappearance of the larger fragment, whereas the amount of the smaller fragment remained fairly constant. These results suggest that CPP32 cleaves MDM2 preferentially at a single site, giving rise to two fragments. The sizes of these fragments suggest that this initial cleavage site corresponds to the consensus site located nearest to either the amino or carboxyl terminus of MDM2 (DLKD or DVPD), rather than to one of the potential cleavage sites in the middle of the MDM2 sequence (Fig. 2). The larger fragment then appears to be degraded as a result of cleavage at the multiple additional DXXD sites present in MDM2. This degradation of the larger fragment in vitro is consistent with the absence of any detectable fragment of MDM2 in apoptotic cell lysates (Fig. 1B).

We then tested MDM2 cleavage by other members of the ICE protease family. ICE did not cleave MDM2 in vitro (Fig. 4B, ICE lane). In contrast, two other members of the CPP32 subfamily, Mch2 (caspase-6) and Mch3 (caspase-7) (7–9), were able to cleave MDM2, producing fragments the same size as those produced by apoptotic cytosol or purified CPP32 (Fig. 4B, Mch3 and Mch2 lanes). In addition, Mch2 yielded a third major cleavage product with apparent molecular mass <30 kDa. However, the cleavage of MDM2 by either Mch2 or Mch3 was significantly less efficient than cleavage by CPP32.

These results suggest that there is a limited specificity of MDM2 cleavage for CPP32, and other CPP32 subfamily members (Mch2 and Mch3) also contribute to the cleavage activity. This cleavage of MDM2 by other CPP32-like proteases proba-

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3 K. J. Tomaselli, unpublished observations.
ably accounts for the remaining activity of apoptotic cytosol following immunodepletion of CPP32 (Fig. 3B).

**Cleavage of MDM2 Can Be Prevented by Mutation of Aspartate 359**—Among the potential cleavage sites in MDM2, the conserved sequence between amino acids 356–359 (DVPD) appeared to be the most likely primary recognition site for CPP32, with cleavage after aspartate 359. This site is almost identical to those in the 70-kDa component of the U1 ribonucleoprotein (DGPD) (10) and in the sterol-regulatory element binding protein 2 (DEPD) (12). Moreover, cleavage at this site would yield two fragments similar in size to those identified in vitro. We therefore introduced a mutation into MDM2, replacing aspartate 359 with glutamic acid (D359E). The mutated MDM2 was resistant to cleavage either by apoptotic cytosol or by purified CPP32 (Fig. 5A), identifying aspartate 359 as the primary site of CPP32 cleavage.

To determine whether aspartate 359 was also a primary site of MDM2 cleavage in vivo, COS-7 cells were transiently transfected with amino-terminal HA-tagged MDM2 expression plasmids. Transfected cells were deprived of growth factors, and the tagged proteins were detected by immunoblotting with anti-HA antibody. With HA-tagged wild type MDM2, a 60-kDa HA-tagged fragment was observed (Fig. 5B, **HA-mdm2 lanes**). The formation of this fragment was increased following growth factor deprivation, although some cleavage was seen in control cells, probably reflecting apoptosis resulting from toxicity of the lipofectamine transfection procedure. In contrast, no cleavage was detected in cells transfected with HA-tagged D359E mutant MDM2 (Fig. 5B, **HA-D359E-mdm2 lanes**), indicating that this mutation protects MDM2 from cleavage in vivo.

These results identify the oncoprotein MDM2 as a novel substrate for the CPP32-subfamily of apoptotic proteases. As an inhibitor of p53, MDM2 plays a direct role in the regulation of apoptosis. The biological significance of MDM2 as a negative regulator of p53 is illustrated by the amplification and overexpression of MDM2 in a variety of tumors, particularly sarcomas, containing normal p53 genes (24). Overexpression of MDM2 in transfected cells also inhibits both p53-induced apoptosis and cell cycle arrest (27, 28). It thus appears that induction of MDM2 by p53 forms a negative feedback loop that is critical to regulation of the growth suppressive and apoptotic activities of p53. Our results indicate that cleavage of MDM2 by CPP32-like proteases breaks this feedback loop in apoptotic cells, resulting in deregulation of p53 and potentially contributing directly to the process of apoptotic cell death.

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