**Proteolysis of the Mismatch Repair Protein MLH1 by Caspase-3 Promotes DNA Damage-induced Apoptosis**

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Caspases are critical proapoptotic proteases that execute cell death signals by selectively cleaving proteins at Asp residues to alter their function. Caspases trigger apoptotic chromatin degradation by activating caspase-activated DNase and by inactivating a number of enzymes that sense or repair DNA damage. We have identified the mismatch repair protein MLH1 as a novel caspase-3 substrate by screening small pools of a human prostate adenocarcinoma cDNA library for cDNAs encoding caspase substrates. In this report, we demonstrate that human MLH1 is specifically cleaved by caspase-3 at Asp418 in vitro. Furthermore, MLH1 is rapidly proteolyzed by caspase-3 in cancer cells induced to undergo apoptosis by treatment with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or the topoisomerase II inhibitor etoposide, which damages DNA. Importantly, proteolysis of MLH1 by caspase-3 triggers its partial redistribution from the nucleus to the cytoplasm and generates a proapoptotic carboxyl-terminal product. In addition, we demonstrate that a caspase-3 cleavage-resistant D418E MLH1 mutant inhibits its etoposide-induced apoptosis but has little effect on TRAIL-induced apoptosis. These results indicate that the proteolysis of MLH1 by caspase-3 plays a functionally important and previously unrecognized role in the execution of DNA damage-induced apoptosis.

The caspase family of cysteine proteases is an essential effector of the apoptotic cell death program that catalyzes many of the biochemical and morphological events of apoptosis by concerted proteolytic actions on a subset of intracellular proteins (1, 2). Caspases are organized in a proteolytic cascade in which initiator pro-caspases are activated by oligomerization via recruitment to distinct caspase-activating complexes; these apoptotic signals are then amplified by the mitochondria. In the extrinsic pathway, ligands of the tumor necrosis factor (TNF)-α family (e.g. TNF-α, TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand) bind to their death domain-containing receptors, an event that leads to the recruitment of the death domain-containing protein FADD and subsequently to the recruitment and activation of procaspases-8 and -10 (1–5). In the intrinsic pathway, caspase-2 rather than caspase-9 has recently emerged as the likely initiator caspase activated by genotoxic stress such as DNA damage and chemotherapeutic drugs (6–8). In response to these stimuli, pro-caspase-2 is recruited to a large cytosolic complex, the components of which have yet to be identified (but are apparently different from the apoptosome discussed below), resulting in its oligomerization and activation (8). Importantly, the apoptotic signals initiated by death ligands or genotoxic stress are amplified by the mitochondria; caspase-8 (extrinsic pathway) and caspase-2 (intrinsic pathway) trigger the mitochondrial release of cytochrome c and other proapoptotic mediators such as Smac/DIABLO (6, 7, 9–12). Pro-caspase-9 is then activated by oligomerization in the apoptosome, a large cytosolic complex composed of cytochrome c, Apaf-1, ATP, and procaspase-9 (13, 14). Caspases-8, -9, and -10 proteolyze and activate downstream caspases, including caspases-3, -6, and -7, which execute the apoptotic cell death signal by cleaving a number of intracellular protein targets (5, 15, 16).

The oligonucleosomal degradation of chromosomal DNA is one of the defining irreversible features of apoptotic cells that facilitates packaging the fragmented genome into apoptotic bodies (1, 17). Caspases promote DNA fragmentation by a variety of mechanisms. First, at least under certain circumstances, caspases induce the mitochondrial release of apoptosis-inducing factor and endonuclease G, which interact with each other to induce chromosomal DNA fragmentation (18–20). Second, caspases proteolyze and inactivate ICAD (also known as DFF-45), an inhibitor of the caspase-activated DNase (CAD) (21–23). ICAD normally binds to and suppresses the DNase activity of CAD. Caspase cleavage of ICAD releases it from CAD, thereby activating CAD, which degrades chromosomal DNA between nucleosomes. Third, caspases cleave several enzymes that sense or repair damaged DNA, including poly(ADP-ribose) polymerase (PARP), RAD21, RAD51, ATM, the catalytic subunit of the DNA-dependent protein kinase, the Bloom syndrome protein (BLM), and BRCA1 (24–31). In the case of RAD21, caspase cleavage generates a proapoptotic carboxyl-terminal product that is sufficient to induce apoptosis (25, 26). In this way, caspases promote DNA fragmentation by activating apoptotic DNases and by systematically subverting the DNA repair machinery.

Here we report that MLH1, a component of the conserved DNA repair machinery, plays a functionally important and previously unrecognized role in the execution of DNA damage-induced apoptosis.
DNA mismatch repair (MMR) complex, is a novel and specific caspase-3 substrate. Indeed, our results are the first to indicate a linkage between caspases and MMR proteins. The MMR system recognizes and repairs mispaired or unpaired nucleotides that result from errors in DNA replication (32, 33). The mammalian homologue of the Escherichia coli MutS protein form heterodimers (MSH2-MSH6 and MSH2-MSH3) that bind to mismatches and recruit heterodimers of the MutH homologues (MLH1-PM52, MLH1-PM51, or MLH1-MLH3) to base mismatches (32–34). These MLH1-containing heterodimers in turn function as adaptor proteins that link the MutS proteins to the DNA repair/replication machinery, resulting in the excision and repair of the mismatch-containing, newly synthesized DNA strand. Inactivating mutations of MLH1 and MSH2 occur commonly in hereditary nonpolyposis colon cancer and less commonly in other carcinomas, and they result in a “mutator” phenotype characterized by instability of repetitive microsatellite DNA sequences (33, 35–38). MMR proteins have also been implicated in homologous recombination repair of DNA double-strand breaks, in cell cycle checkpoint activation, and in the execution of the apoptotic response to DNA damage induced by alkylating agents and other drugs that modify nucleotides or inhibit topoisomerase II (39–47).

In the present work, we show that MLH1 is rapidly and specifically cleaved by caspase-3 at Asp118 in cells induced to undergo apoptosis by treatment with TRAIL (extrinsic apoptotic pathway) or etoposide (intrinsic apoptotic pathway), a chemotherapeutic drug that inhibits topoisomerase II and induces DNA double-strand breaks (48). Furthermore, we demonstrate that caspase proteolysis of MLH1 induces its partial relocation from the nucleus to the cytoplasm and produces a proapoptotic carboxyl-terminal product. We also show that a caspase cleavage-resistant mutant of MLH1 inhibits apoptosis induced by etoposide but has little effect on TRAIL-induced apoptosis, thereby indicating a novel role for caspase proteolysis of MLH1 in the execution of DNA damage-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs—**cDNAs encoding GFP-tagged full-length MLH1, the amino-terminal caspase cleavage product (N-MLH1), amino acids 1–418, or the carboxyl-terminal caspase cleavage product (C-MLH1), amino acids 419–766) were made by PCR amplification of wild-type human MLH1 (kindly provided by Dr. R. D. Kolodner) using the following oligonucleotide primers: 5’-GGCCCTCGAGGACAAAATGTCCGTTGGTG-G3’ and 5’-G GCCCGGATCCATCTTTCATGTTCGTCGTT-3’ (full-length MLH1), 5’-GGCCGCTTGCGACAAATGTCCGTTGGTG-G3’ and 5’-G GCCGCGGATCCATCTTTCATGTTCGTCGTT-3’ (N-MLH1), or 5’-GGCCGCTCGAGTAGATTTCTAGTGGCAGGGC-3’ and 5’-CCTGCCACTGAAATCTGCTGTATCC-5’ (C-MLH1). Each amplified PCR product was digested with Xhol and SacI and subcloned into pEGFP-N1 (Clontech). All cDNAs were verified by DNA sequencing.

**Small Pool Expression Cloning—**Small pool expression cloning to identify cDNAs encoding caspase substrates was performed as described previously (25, 49–51) with the following exceptions. Small pools (48 cDNA/pool) of a human prostate adenocarcinoma cDNA library (Invitrogen, catalogue product 11597010) were used to make 35S-labeled protein pools with the Tnt SP6-coupled transcription/translation system (Promega) as described previously (49, 50). 35S-Labeled protein pools were incubated with buffer control or 25 ng of caspase-1, -2, -3, or -8 for 1 h at 37 °C; the cleavage reactions were then separated by SDS-PAGE and visualized by autoradiography as described previously (49, 50). Single cDNAs encoding putative caspase substrates were isolated by systematically subdividing small cDNA pools and retesting the corresponding 35S-labeled protein pools as described previously (49, 50).

**Caspase Cleavage of MLH1 in Vitro—**35S-Labeled full-length human MLH1 was incubated with buffer or 2.5 or 25 ng of caspase-1, -2, -3, -4, -6, -7, or -8 for 1 h at 37 °C, and the reactions were analyzed as described previously (50, 52). A potential caspase-3 cleavage site in MLH1 (Asp118) was specifically altered to a Glu residue with the QuickChange site-directed mutagenesis kit (Stratagene) using the primers 5’-GAG-

**RESULTS**

**Identification of MLH1 as a Caspase-3 Substrate by Small Pool Expression Cloning—**We have described recently a small pool expression cloning strategy to screen cDNA libraries for cDNAs encoding caspase substrates (25, 49–51). In this report, small pools (48 cDNAs/pool) of a human prostate adenocarcinoma cDNA library (Invitrogen) were transcribed and translated in vitro in the presence of [35S]methionine, and the corresponding 35S-labeled protein pools were incubated with recombinant caspases. As shown in Fig. 1A, a ~66-kDa protein (indicated by an asterisk) present in 35S-labeled protein pool 10 was specifically cleaved by caspase-3 (C3), but not by the other caspases tested (caspase-1, -2, or -8), into two products, ~45 and 24 kDa in size (indicated by arrows). The enzymatic activity of each protease was verified with a known substrate (data not shown). To isolate the putative caspase-3 substrate present in protein pool 10, cDNA pool 10 was further subdivided into smaller pools, and the corresponding 35S-labeled protein pools were reincubated with caspase-3. This process was repeated until a single cDNA encoding a ~66-kDa protein cleaved by caspase-3 into the appropriately sized fragments was identified (Fig. 1B). This cDNA was sequenced and found to be a partial MLH1 cDNA (36, 37).

**MLH1 Is Specifically Proteolyzed by Caspase-3 at Asp118 in Vitro—**To determine whether the full-length MLH1 protein is cleaved by caspases in vitro, we incubated 35S-labeled full-length human MLH1 with recombinant caspases. As shown in Fig. 2A, 35S-labeled MLH1 was selectively proteolyzed by caspase-3 into two major cleavage products, which were ~45 and 40 kDa in size (indicated by arrows). In contrast, none of the other caspases (caspase-1, -2, -6, -7, or -8) examined cleaved
MLH1. The activity of each caspase was confirmed using a known substrate (data not presented). To identify the caspase cleavage site in human MLH1, we substituted the Asp residue (Asp\(^{118}\)) at a potential caspase-3 cleavage site (Asp-Lys-Thr-\(°\)) with a Glu residue. Unlike WT MLH1 (Fig. 2B, left panel), the \(^{35}\)S-labeled D418E MLH1 protein was not cleaved by caspase-3 (right panel), indicating that Asp\(^{118}\) is the caspase-3 cleavage site in vitro.

**MLH1 Is Cleaved by Caspase-3 in Cancer Cells Undergoing Apoptosis**—To determine whether MLH1 is cleaved in cancer cells during the induction of apoptosis, we treated human PC-3 prostate carcinoma cells with 2 \(\mu\)g/ml TRAIL for 0–16 h or human TSU-Pr1 bladder carcinoma cells with 50 \(\mu\)M etoposide for 0–36 h. As demonstrated in Fig. 3A, MLH1 was rapidly proteolyzed into a ~45-kDa product (indicated by an arrow) in cells treated with TRAIL (within 4 h) or with etoposide (within 12 h). The caspase substrate protein kinase C\(\delta\) (55) was used as a positive control in these studies and was cleaved into its characteristic fragment (Fig. 3A, indicated by an arrow) in apoptotic cells. Importantly, the size of the apoptotic MLH1 cleavage product was similar to the larger of the two products generated by caspase-3 in vitro. Furthermore, MLH1 proteolysis occurred at a similar time after exposure to apoptotic stimuli as did caspase-3 activation (seen as a reduction in procaspase-3 levels because of proteolytic processing). Taken together, these findings suggest that MLH1 might be cleaved by caspase-3 in apoptotic cells. Consistent with this notion, the broad spectrum caspase inhibitor Z-VAD-fmk potently suppressed MLH1 cleavage in PC-3 cells treated with TRAIL or etoposide (Fig. 3B, upper panels) or in TSU-Pr1 cells treated with these same apoptotic stimuli (lower panels). To evaluate specifically the role of caspase-3 in the apoptotic proteolysis of MLH1, we treated caspase-3-deficient MCF-7 breast carcinoma cells (56) with 10 ng/ml TNF-\(\alpha\) and 1 \(\mu\)g/ml cycloheximide for 0–12 h. As shown in Fig. 3C, MLH1 was not cleaved in caspase-3-deficient MCF-7 cells treated with TNF-\(\alpha\), while RAD21, a known substrate of multiple caspases (25), was proteolysed in these cells. In addition, MLH1 was not cleaved in MCF-7 cells treated with etoposide (data not shown). These results indicate that MLH1 is a specific proteolytic target of caspase-3 that is cleaved in cancer cells in response to diverse apoptotic stimuli.

**Caspase Proteolysis of MLH1 Triggers Its Partial Relocalization from the Nucleus to the Cytoplasm and Generates a Pro-apoptotic Carboxyl-terminal Product**—To begin to assess the functional consequences of MLH1 proteolysis by caspase-3, we transiently transfected PC-3 prostate carcinoma cells with GFP-tagged constructs encoding WT MLH1 or the amino-terminal (amino acids 1–418, N-MLH1) or carboxyl-terminal (amino acids 419–756, C-MLH1) MLH1 caspase cleavage products. As shown in Fig. 4A (upper panels), WT MLH1 and C-MLH1 were expressed in the nuclei of transfected cells, while N-MLH1 was found in both the nucleus and the cytoplasm. The nuclei (Fig. 4A, lower panels) of cells transfected with WT MLH1 or N-MLH1 construct were intact (i.e. non-apoptotic). Although most cells transfected with C-MLH1 also had intact nuclei, a subset of these cells had fragmented, apoptotic nuclei (Fig. 4B, apoptotic nucleus indicated by an arrow). Indeed, transient transfection of PC-3 cells (which express MLH1) or MLH1-deficient RKO.3 cells (RKO colorectal carcinoma cells stably transfected with empty pcDNA3 vector (57)) with each of the MLH1 constructs revealed that C-MLH1 was proapoptotic (Fig. 4C). In contrast, WT MLH1 and N-MLH1 did not induce apoptosis above background levels observed in vector-transfected cells. Overall, these results indicate that caspase cleavage of MLH1 alters its subcellular localization and produces a proapoptotic carboxyl-terminal product.

**A Caspase Cleavage-resistant MLH1 Mutant Protects against Apoptosis Induced by Etoposide**—To examine whether the pro-
teolytic cleavage of MLH1 by caspase-3 is necessary for the execution of apoptosis, we transiently transfected PC-3 cells with GFP-vector or cDNAs encoding GFP-tagged WT or D418E mutant MLH1. After 24 h, transfected PC-3 cells were treated with 2 μg/ml TRAIL for 1 h or 50 μM etoposide for 6 h (transient transfection sensitizes cells to apoptosis induction), and GFP-positive cells were scored for apoptotic nuclei. As shown in Fig. 5, vector- and WT MLH1-transfected cells were sensitive to
apoptosis induction by TRAIL or etoposide. In contrast, mutant D418E MLH1-expressing cells were partly protected against etoposide-induced apoptosis, while their apoptotic response to TRAIL was largely unaffected. These results indicate that MLH1 proteolysis is a functionally important event in the execution of DNA damage-induced apoptosis.

**DISCUSSION**

We have identified the mismatch repair protein MLH1 as a novel, functionally relevant substrate of caspase-3 that is rapidly proteolyzed in cells induced to undergo apoptosis by stimuli that engage the extrinsic (TRAIL) or the intrinsic (etoposide) apoptotic pathways. MLH1 is selectively cleaved by caspase-3 (but not by other caspases tested) in *vitro* and is not cleaved in apoptotic caspase-3-deficient MCF-7 cells. These results indicate that MLH1 is a specific substrate of caspase-3, one of the major downstream executioner caspses (1, 2). Intriguingly, experiments performed with cells derived from caspase-3 knockout mice or with caspase-3-deficient MCF-7 cells have revealed that caspase-3 is required for apoptotic chromatin condensation and DNA fragmentation, perhaps because ICAD proteolysis, and therefore CAD activation, is impaired in the absence of caspase-3 (16, 56, 58). MLH1, then, can be added to a short list of caspase-3-specific substrates, which...
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...defective activation of the p53/p73 apoptotic response. MMR proteins, then, are key components of the apoptotic response to DNA damage. Indeed, PMS2 directly binds/stabilizes p73 and enhances its proapoptotic activity, suggesting that MMR proteins may target components of the cell death machinery to damaged DNA and trigger their activation (64). Additional support for this notion comes from the observation that MLH1 binds with the proapoptotic oncoprotein c-MYC (66). Furthermore, MLH1 is a component of the BRCA1-associated genome surveillance complex (which also includes MSH2, MSH6, ATM, BLM, RAD50, and other components) that has been implicated in the detection and repair of damaged DNA (67). Interestingly, several of the components of this complex (BRCA1, BLM, ATM, and MLH1) are cleaved by caspases (28, 30, 31). Like MLH1, BRCA1 cleavage by caspases promotes DNA damage-induced apoptosis (31). Finally, caspase proteolysis of RAD21, a cohesin component that repairs DNA double-strand breaks, produces a carboxyl-terminal cleavage product that induces apoptosis (25, 26).

The results reported here indicate for the first time that proteolysis of MLH1 plays a similarly important role in activating its proapoptotic function in response to DNA damage, perhaps by deregulating its interactions with other MMR proteins, components of the BRCA1 complex, or apoptotic proteins. Taken together, these observations suggest that components of the DNA repair machinery are converted to apoptotic executors by caspase cleavage, an elegant and efficient strategy to signal DNA damage-induced apoptosis.

Acknowledgments—We are indebted to Dr. R. Talanian for providing recombinant caspases, to Dr. R. D. Kolodner for providing the MLH1 cDNA, and to Dr. D. Boothman for providing cancer cell lines.

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