DNA Ligase III Is Degraded by Calpain during Cell Death Induced by DNA-damaging Agents*

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Laura Bordone† and Colin Campbell‡

From the Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

A yeast two-hybrid screen identified the regulatory subunit of the calcium-dependent protease calpain as a putative DNA ligase III-binding protein. Calpain binds to the N-terminal region of DNA ligase III, which contains an acidic proline, aspartate, serine, and threonine (PEST) domain frequently present in proteins cleaved by calpain. Recombinant DNA ligase III was a substrate for calpain degradation in vitro. This calpain-mediated proteolysis was calcium-dependent and was blocked by the specific calpain inhibitor calpeptin. Western blot analysis revealed that DNA ligase III was degraded in human fibrosarcoma HT1080 cells following exposure to γ-radiation. The degradation of DNA ligase III was prevented by pretreatment with calpeptin, which protected irradiated cells from death. Calpeptin treatment also blocked 9-amino camptothecin-induced DNA ligase III proteolysis and simultaneously protected the cells from death. HT1080 clones expressing a modified DNA ligase III was more resistant to killing by γ-radiation. HT1080 clones expressing a modified DNA ligase III proteolysis and simultaneously protected the cells from death. Calpeptin treatment also blocked 9-amino camptothecin-induced DNA ligase III proteolysis and simultaneously protected the cells from death. HT1080 clones expressing a modified DNA ligase III was more resistant to killing by γ-radiation. HT1080 clones expressing a modified DNA ligase III proteolysis and simultaneously protected the cells from death. Calpeptin treatment also blocked 9-amino camptothecin-induced DNA ligase III proteolysis and simultaneously protected the cells from death.

During programmed cell death, or apoptosis, cells are destroyed via a highly orchestrated series of intracellular events. While this process was discovered during studies of development, apoptosis also plays a critical role in a number of processes that occur in the adult organism, including tissue homeostasis and elimination of virus-infected and tumor cells (1–5). A variety of signals can trigger programmed cell death involving tumor necrosis factor-α (6), Fas ligand (7), growth factor withdrawal (8), and DNA-damaging agents (9). A key feature of apoptosis is the induction of intracellular protease activity. Biochemical and genetic studies have determined that proteolytic cleavage of key cellular proteins by these enzymes is an essential process in the apoptotic pathway (10, 11).

The most studied of these proteases belong to the caspase gene family and are encoded by homologs of the Caenorhabditis elegans ced 3 gene (1, 12). However, mammalian apoptosis also involves the activation of proteases encoded by the cathepsin (13) and calpain gene families (14–16). Activation of members of the caspase and cathepsin gene families occurs via proteolytic cleavage of inactivezymogen forms (11, 17). In contrast, activation of calpains requires elevated levels of cellular calcium (Ca²⁺), although these proteins are further activated, and have their Ca²⁺ requirement reduced by analysis that occurs following the initial Ca²⁺-dependent activation (18–21).

Proteases involved in apoptosis appear to hydrolyze target proteins at a limited number of specific recognition sites. For example, most members of the caspase family cleave proteins at the C-terminal end of a 4-amino acid sequence containing a terminal aspartic acid residue (11, 22), whereas calpains specifically cleave proteins containing regions rich in proline, aspartate, serine, and threonine residues called PEST motifs (23, 24). Proteolytic cleavage can activate the target protein, as in the case of autolysis of calpains, caspase-activated deoxyribonuclease, or MEKK-1 kinase (25, 26). However, in many cases proteolysis leads to inactivation of the protein (27, 28).

The range of cellular proteins that undergoes specific degradation during apoptosis is extensive and includes cell cycle regulatory proteins (i.e. p21 (29)), cytoskeletal elements (i.e. actin, fodrin, and lamin (30–32)), and signal transduction proteins (i.e. protein kinase C, Akt1 (33–35)).

During apoptosis triggered by DNA damage, caspase 3 hydrolyzes a number of proteins involved in DNA damage recognition and repair. For example, the catalytic subunit of the DNA-dependent protein kinase is cleaved by caspase 3 following exposure to ionizing radiation (IR) (36). In addition, it has been shown that poly(ADP-ribos)e polymerase (37) and the ataxia telangiectasia-mutated protein (38) are cleaved by this enzyme in cells exposed to IR. The Bloom syndrome protein is cleaved by caspase 3 in Jurkat cells undergoing apoptosis induced by a topoisomerase inhibitor (39). Finally, it has been shown that the DNA repair enzyme Rad51 is cleaved by caspase 3 in cells that have been exposed to γ-radiation (40). Interestingly, cells that overexpressed a protease-resistant form of Rad51 were resistant to the cytotoxic effects of IR, suggesting that the DNA repair activity mediated by this enzyme could antagonize the cell death signal, thereby promoting cellular survival (40).

This hypothesis suggests that other DNA repair enzymes are likely to be degraded in response to treatment with DNA-damaging agents and that alleles of genes that encode pro-

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‡ To whom correspondence should be addressed: Dept. of Pharmacology, University of Minnesota Medical School, 6-120 Jackson Hall, 321 Church St., SE, Minneapolis, MN 55455. Tel.: 612-625-8986; Fax: 612-625-8408; E-mail: campb034@umn.edu.

The abbreviations used are: PEST, proline, aspartate, serine, and threonine; BAC, 9-amino camptothecin; IR, ionizing radiation; WT, wild-type; HA, hemagglutinin; PARP, poly(ADP-ribose) polymerase; FL, full length; GFP, green fluorescent protein; BSA, bovine serum albumin; TBS, Tris-buffered saline; Gy, gray; ANOVA, analysis of variance; MOD, modified.
DNA Ligase III Degradation during Programmed Cell Death

The results described herein support this hypothesis. We demonstrate that DNA ligase III is a substrate for calpain-mediated proteolysis. Calpain binding to and degradation of DNA ligase III is dependent upon a PEST (23, 24) sequence that is present in the N-terminal portion of the latter protein. We further determined that calpain degrades DNA ligase III during cell death induced by a number of DNA-damaging agents and that expression of a recombinant DNA ligase III protein that was resistant to calpain-mediated proteolysis protected cells from killing by these agents. These findings suggest that calpain-mediated proteolysis of DNA ligase III plays an essential role in DNA damage-induced cell death.

EXPERIMENTAL PROCEDURES

Bacterial and Saccharomyces cerevisiae Strains and Eukaryotic Cells—Escherichia coli DH10B cells were used for subcloning of cDNA and for amplification of plasmids recovered from yeast. For yeast two-hybrid system screening the reporter strains PJ69-2A (MATα, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, UAS2-GAL1-αACT-HIS3, GAL1-αACT-GAL2-TATA-ADE2) and Y187 (MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met2, URA3: GAL1-αACT-GAL2-TATA-lacZ) were used. Human HT1080 fibrosarcoma cells (41) were grown in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 9% fetal bovine serum (Invitrogen). Mouse sarcoma cells (41) were grown in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 9% fetal bovine serum (Invitrogen). reverse transcriptase-PCR was used to amplify nucleotides 3102 of the human DNA ligase III cDNA from HT1080 cells. The full-length cDNA of wild-type and PEST-modified DNA ligase III were cloned in-frame with the DNA binding domain of Gal4, which recognizes amino acids 1–1411 of DNA ligase III (42). The vector pAS-FL was used at a 1:1000 dilution, or a rat monoclonal anti-HA high affinity antibody (Santa Cruz Biotechnology) incubated for 1 h at room temperature with goat anti-mouse or anti-rat IgG conjugated with alkaline phosphatase substrate (SigmaFast, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, Sigma). The reaction was then stopped by rinsing the membrane with water, and the blot was scanned and quantified with the Molecular Analyst program.

Identification of DNA Ligase III PEST Sequence—The sequence spanning amino acids 145–158 (KPNNAGEAPAAPPAP) of DNA ligase III was recognized as a PEST sequence by the PEST-FIND algorithm developed by Rogers and colleagues (45). (www.at.embnet.org/hbiv/embnet/PESTfind).

Site-directed Mutagenesis of DNA Ligase III PEST Sequence—Oligonucleotide site-directed mutagenesis of the PEST sequence of human DNA ligase III was performed using the method of Kunkel et al. (46). Mutagenic oligonucleotides were used to alter the coding region corresponding to the PEST domain of DNA ligase III. The modified cDNA encoded a DNA ligase III protein in which serine and threonine residues present in the PEST region (KPNNSGEAPSSPTP) of DNA ligase III were replaced by alanines (KPNNAGEAPAAPPAP). In the process a new SacII restriction site was created. Clones bearing the desired mutations were identified by restriction digesting using SacII and by DNA sequence analysis. This modified DNA ligase III cDNA was subcloned into the pAS2-1 vector, creating plasmid pAS-MOD. Yeast were then co-transformed with this plasmid and pACT-CAL, the calpain-encoding plasmid identified in the original screen.

Morphological Examination of Irradiated Cells—HT1080 cells expressing the WT or PEST-modified DNA ligase III were transiently transfected (Superfect; Qiagen Inc., Valencia, CA) with a vector encoding the green fluorescent protein (GFP; CLONTECH, Palo Alto, CA). Following a 48-h incubation, the cells were exposed to 5 Gy of IR or were mock-irradiated. One, two, and four hours later fluorescent microscopy was performed. The samples were coded to eliminate biased scoring, and 100 cells were examined to determine the relative percentage of living (flat, adherent) and dying (rounded, shrunken) cells present before and after irradiation. The results presented in Fig. 7 were obtained by subtracting the former value from the latter.
DNA Ligase III Interacts with Calpain—A full-length cDNA of the human DNA ligase III gene was used as “bait” in a yeast two-hybrid experiment. Slightly more than 2 million yeast transformants expressing clones obtained from a human cDNA liver library were screened to identify potential DNA ligase III-binding proteins. One of the positive clones harbored a cDNA that upon sequence analysis was found to encode a portion of the small subunit of the calcium-dependent protease calpain (amino acids 142–268).3

The N Terminus of DNA Ligase III Interacts with Calpain—To determine the calpain-binding region of the DNA ligase III protein, different fragments of the DNA ligase III cDNA were subcloned into the two-hybrid vector pAS2-1 thereby creating a series of ligase III-Gal4 DNA binding domain fusion genes (pAS-FL, pAS-A, pAS-B, and pAS-C; see “Experimental Procedures”). As Fig. 1A illustrates, the different cDNA fragments encode one or more of the domains known to be present in the DNA ligase III protein. Plasmids encoding these fusion proteins were co-transformed into yeast that expressed the Gal4-calpain regulatory subunit “prey” fusion protein that was identified in the original two-hybrid screen (encoded by plasmid pACT-CAL). As Fig. 1B indicates, bait-prey interactions were only detected in clones that expressed either the full-length DNA ligase III or construct A, indicating that a calpain-binding domain of DNA ligase III is located within the N-terminal portion of the protein.

DNA Ligase III Has a PEST Sequence—Many known calpain substrates contain a so-called PEST domain (23, 24). PEST domains are regions of a protein rich in proline, glutamate/aspartate, serine, and threonine residues that are flanked by basic amino acid residues.

The computer algorithm PEST-Score can be used to determine whether a particular protein contains a PEST sequence. The algorithm assigns a score to any PEST sequence identified, and a PEST score greater than 0 suggests that the protein is a likely calpain substrate (45). Computer analysis revealed the presence of a PEST sequence spanning amino acids 145–158 (single letter amino acid code: KPNNSGEAPSSPTP, Fig. 1A) of DNA ligase III. The PEST score for this sequence was +10.47, indicating a strong likelihood that this is an authentic PEST domain. Interestingly, this PEST sequence is located within the portion of the DNA ligase III protein that interacts with calpain in the yeast two-hybrid analysis.

Human DNA Ligase III Is a Calpain Substrate—A series of experiments were performed to determine whether DNA ligase III is a substrate for calpain-dependent proteolysis. First, nuclear protein extracts from human HT1080 cells were incubated with calpain under a variety of conditions, resolved by SDS-PAGE, and DNA ligase III levels monitored by Western blot analysis using an anti-DNA ligase III antibody. As Fig. 2A indicates, although addition of calpain alone had no effect on DNA ligase III, addition of calcium (750 μM) plus calpain resulted in complete degradation of DNA ligase III. As would be

3 The amino acid sequence for the human calpain small subunit can be accessed through NCBI Protein Database under accession number P04832.
DNA ligase III is degraded in vitro by calpain. A, nuclear extracts (20 μg) from HT1080 were incubated at 30 °C for 30 min with purified calpain (1 μg) in the presence or absence of calcium (750 μM) or EGTA (1.5 mM). Electrophoresis was performed using a 7.5% polyacrylamide gel. Western blot analysis was performed using an antibody that recognizes endogenous DNA ligase III (LIG3). B, nuclear extracts (20 μg) from HT1080 overexpressing a recombinant HA-DNA ligase III protein were incubated at 30 °C for 30 min with purified calpain (1 μg) in the presence or absence of calcium (750 μM) or EGTA (1.5 mM). Calpeptin (520 nM) was used to prevent calpain activation. Electrophoresis was performed using a 7.5% polyacrylamide gel. Immunoblot analysis was performed using an antibody that recognizes the HA epitope. C, 15 μl of in vitro transcribed and translated DNA ligase III was incubated at 30 °C for 30 min with purified calpain (1 μg) in the presence or absence of calcium (750 μM) as indicated. EGTA (1.5 mM) and calpeptin (520 nM) were added where indicated. Electrophoresis was performed using a 7.5% polyacrylamide gel. Western blot analysis was performed using an antibody that recognizes the HA epitope.

Expected, when EGTA (a calcium chelator) was included along with calcium and calpain, no proteolysis of DNA ligase III was observed. Interestingly, addition of calcium alone also resulted in partial proteolysis of DNA ligase III, indicating that HT1080 cells possess an endogenous calcium-dependent protease that is capable of degrading DNA ligase III.

Similar experiments were performed using nuclear protein extracts prepared from a HT1080 cell line that overexpressed an HA-tagged DNA ligase III gene (see “Experimental Procedures”). As Fig. 2B indicates, the HA-tagged DNA ligase III protein present in extracts prepared from these cells was degraded in the presence of calcium and calpain. When the specific calpain inhibitor calpeptin was included in the reaction, DNA ligase III was protected from degradation. As was seen with the endogenous DNA ligase III, addition of calcium alone resulted in partial degradation of the HA-tagged DNA ligase III protein.

The results presented in Fig. 2, A and B, are consistent with the hypothesis that DNA ligase III is a substrate for calpain proteolysis. However, these results also support the hypothesis that addition of calpain activates a second protease that cleaves DNA ligase III. To distinguish between these two hypotheses, recombinant DNA ligase III was prepared by in vitro transcription and translation and was incubated with calpain in the presence or absence of calcium. As Fig. 2C shows, addition of calpain and 750 μM calcium resulted in DNA ligase III degradation. This effect was blocked by addition of EGTA or calpeptin. These results indicate that DNA ligase III is a calpain substrate. This finding in turn suggests that the degradation of DNA ligase III observed in Fig. 2, A and B, is likely due to the direct action of calpain. However, these results do not rule out the possibility that other proteases could be responsible for the calpain-dependent degradation of DNA ligase III shown in Fig. 2, A and B.

Characterization of a DNA Ligase III Mutant with a Modified PEST Sequence—The results presented thus far are consistent with the hypothesis that DNA ligase III binds to and is degraded by calpain due to the presence of a PEST domain near the N terminus of the protein. To test this hypothesis, site-directed mutagenesis was performed to modify the portion of the HA-tagged DNA ligase III cDNA that encoded the PEST sequence. The resulting cDNA encoded a modified DNA ligase III protein in which three of the serine residues and the threonine residue within the DNA ligase III PEST domain were replaced by alanine residues (see the “Experimental Procedures”). Analysis by PEST score indicated that the DNA ligase III protein encoded by this mutant cDNA did not possess a recognizable PEST domain.

The modified DNA ligase III cDNA was cloned into an appropriate vector (pAS-MOD), and a two-hybrid analysis was performed in a yeast strain that expressed the calpain prey clone isolated from the original screen. As a control, a parallel experiment was performed with a yeast clone expressing the wild-type (WT) DNA ligase III cDNA (pAS-A). Although there was a robust interaction between the calpain fragment and the WT DNA ligase III, there was no detectable interaction between calpain and the DNA ligase III protein containing the modified PEST sequence (data not shown). Western blot analysis revealed that the respective yeast transformant clones harbored similar levels of the WT and PEST-modified form of DNA ligase III, suggesting that lack of interaction between calpain and the PEST-modified DNA ligase III was not due to instability of the latter protein (data not shown).

Surprisingly, it has not yet been shown directly that PEST sequences are directly involved in the binding of calpain to any of its substrate molecules. However, the results described above suggest that binding of calpain to DNA ligase III is dependent upon a functional PEST sequence, which is not present on the modified DNA ligase III clone. One clear prediction of this model is that the modified DNA ligase III protein should be less sensitive to calpain-mediated degradation than is the WT DNA ligase III protein. To test this hypothesis, extracts were prepared from HT1080 cells expressing WT and modified (MOD) DNA ligase III proteins. These extracts were then incubated in the presence (+) or absence (−) of calpain and calcium, and Western blot analysis was performed using an anti-HA antibody. Fig. 3A depicts a representative experiment indicating that while the WT DNA ligase III protein was extremely sensitive to proteolysis by calpain, the modified DNA ligase III was only partially hydrolyzed. This experiment was repeated several times, and the extent of DNA ligase III degradation was quantitated by using scanning densitometry. As Fig. 3B indicates, there was a significant difference between the relative sensitivity of the WT and PEST-modified forms of DNA ligase III to degradation by calpain (p < 0.001, n = 4, ANOVA).
DNA Ligase III Degradation during Programmed Cell Death

We also used a clonogenic assay to examine the ability of calpeptin pretreatment to confer a long term survival benefit on irradiated cells. HT1080 cells that expressed an HA-tagged DNA ligase III were pretreated with calpeptin and then exposed to IR. As Fig. 4E shows, cells pretreated with calpeptin were completely resistant to killing by 1.25 or 2.5 Gy of IR. In contrast, cell death was observed in irradiated cells that had been treated with the calpeptin vehicle alone. This finding, which is statistically significant \( p < 0.05 \), confirms that pretreatment with calpeptin protects cells from death induced by IR.

The results presented in Fig. 4 indicate that calpain plays an essential role in mediating both IR- and 9AC-induced cell death. In both cases, cell death was associated with degradation of DNA ligase III. These results are consistent with the hypothesis that DNA ligase III proteolysis is required for cell death. However, it is equally plausible that degradation of calpain substrates other than DNA ligase III is responsible for the cell death caused by these agents.

Creation of HT1080 Cell Lines Expressing WT or PEST-modified Alleles of DNA Ligase III—If calpain-dependent degradation of DNA ligase III were an essential feature of cell death, cells expressing the calpain-resistant version of this protein should be less sensitive to the cytotoxic effects of IR and 9AC than cells expressing WT DNA ligase III. To test this hypothesis, we created transgenic HT1080 cells that overexpressed the modified or WT DNA ligase III protein. Before examining the sensitivity of these cell lines to IR and 9AC, it was essential to characterize them, because differences in the level of DNA ligase III protein could explain any differences in their relative sensitivity to DNA-damaging agents.

We first examined whether the two cell lines expressed similar levels of the two DNA ligase III alleles. As Fig. 5A shows, Western blot analysis using an antibody that recognizes the HA epitope reveals that the two cell lines had similar levels of DNA ligase III cross-reacting protein. Coomassie staining of total proteins showed that this result was not due to different loading of proteins on the gel (Fig. 5B). However, simply demonstrating that the two cell lines possess similar levels of DNA ligase III protein is not sufficient, because it is conceivable that the PEST-modified DNA ligase III protein possesses greater DNA repair activity than does the WT DNA ligase III protein. If this were the case, the transgenic clone expressing the former allele could be inherently less sensitive to killing by DNA-damaging agents simply due to their enhanced ability to repair the initial DNA damage. To examine this possibility, the level of DNA ligase III enzymatic activity present in the transgenic cell lines was determined. This was accomplished by performing nick-sealing assays on whole cell extracts prepared from these cell lines. This assay measures the ability of whole cell protein extracts to ligate a substrate that contains a nicked duplex DNA. The ligase substrate was prepared by annealing two oligonucleotides (one of which was radioactively labeled) adjacent to each other on single-stranded M13 phage DNA. This substrate was incubated with whole cell extracts prepared from HT1080 cells that overexpressed the WT or modified DNA ligase III, and extracts were prepared from cells that had been transfected with the empty pREP4 expression vector. A time course of ligation of the two oligonucleotides was performed, and the formation of the 32-mer product was determined by electrophoresis on a denaturing polyacrylamide gel (Fig. 5C). Scanning densitometry was performed on this image, as well as on images obtained from three additional independent experiments to quantitate the amount of product formed. This analysis revealed that, as expected, extracts from both the WT and modified DNA ligase III-expressing cells had nearly twice as

**Fig. 3.** DNA ligase III protein with a modified PEST domain is significantly more resistant to degradation by calpain compared with WT DNA ligase III. A, 30 μg of whole cell lysates from HT1080 cells that overexpress wild-type (WT) and PEST-modified (MOD) DNA ligase III (LIG3) were incubated with (+) or without (−) calcium (750 μM) and purified calpain (1 μg). Electrophoresis was performed using a 7.5% polyacrylamide gel. Western blot analysis was performed using an antibody that recognizes the HA epitope. B, levels of DNA ligase III were quantified by densitometry. The bar represents % DNA ligase III immunoreactivity (100% = immunoreactivity without calpain). Analysis was performed using the Molecular Analyst software. Results represent mean ± S.E. of three independent experiments. *, \( p < 0.05 \) compared with PEST modified DNA ligase III (MOD). Black bars, minus calpain and calcium; white bars, minus calpain and calcium.

Exposure to 9AC and IR Leads to Calpain-dependent Degradation of DNA Ligase III and Cell Death—It is known that a number of DNA repair enzymes are degraded in cells undergoing programmed cell death following treatment with topoisomerase inhibitors (see Introduction). The results presented thus far suggest that calpain could degrade DNA ligase III in HT1080 cells undergoing programmed cell death. To test this hypothesis, HT1080 cells were incubated for 4 h with the topoisomerase I inhibitor 9AC, and the level of DNA ligase III protein was monitored using Western blot analysis. As Fig. 4A indicates, there was a time-dependent loss of DNA ligase III from lysates prepared from 9AC-treated cells. However, substantially less DNA ligase III degradation was detected in lysates prepared from 9AC-treated cells that had been preincubated with calpeptin. As Fig. 4B indicates, pretreatment with calpeptin significantly reduced the cytotoxic effect of 9AC on HT1080 cells \( p < 0.05, n = 4 \) ANOVA.

The effect of IR on DNA ligase III levels in HT1080 cells was also examined. Cell death following exposure to this agent was also associated with DNA ligase III degradation (Fig. 4C). Furthermore, cell death was prevented by pretreatment of the cells with calpeptin (Fig. 4D). It is noteworthy that pretreatment with calpeptin completely protected cells from death caused by IR, whereas this compound provided only partial protection against the cytotoxic effects of 9AC.

**Fig. 4.** Scanning densitometry was performed on this image, as well as on images obtained from three additional independent experiments to quantitate the amount of product formed. This analysis revealed that, as expected, extracts from both the WT and modified DNA ligase III-expressing cells had nearly twice as much...
much DNA ligase III activity as did extracts prepared from control cells (1.8 ± 0.6- and 2.0 ± 0.4-fold, respectively). However, there was no significant difference between the level of DNA ligase III activity seen in extracts prepared from either of the two transgenic cell lines (ANOVA).

A Calpain-resistant Form of DNA Ligase III Protects HT1080 Cells from Death Induced by IR and 9AC—By having established that the cell lines expressing the WT and PEST-modified DNA ligase III alleles possess similar levels of DNA ligase activity, we examined the relative sensitivity to these two cell lines to the cytotoxic effects of IR using a clonogenic assay. These experiments revealed that HT1080 cells expressing the PEST-modified allele of DNA ligase III were significantly more resistant to killing by IR than were cells that expressed the WT allele of DNA ligase III (Fig. 6).

An additional series of experiments was performed to evaluate further the radio-protective effect conferred by the modified version of DNA ligase III. In these experiments, cells expressing either WT or modified DNA ligase III were transiently transfected with a vector encoding the green fluorescent protein (GFP). Following a 48-h incubation, the cells were transiently transfected with a vector encoding the green fluorescent protein (GFP). Following a 48-h incubation, the cells wereMeans ± S.E. of four independent experiments. *, p < 0.05 (ANOVA) compared with unirradiated cells.

Results represent the mean ± S.E. of four independent experiments. *, p < 0.05 (ANOVA) compared with unirradiated cells. E, HT1080 cells expressing the HA-tagged WT DNA ligase III were pretreated with 100 μM calpeptin (black bars) or calpeptin vehicle (white bars) for 1 h at 37°C. Cells were then exposed to 0, 1.25, or 2.5 Gy of IR, and whole cell lysates were collected and counted, and percent survival was calculated. Results represent the mean ± S.E. of four independent experiments. *, p < 0.05 (ANOVA) compared with unirradiated cells.

Results represent the mean ± S.E. of three independent experiments. *, p < 0.05 (ANOVA) compared with unirradiated cells.
cell line contribute to the resistance phenotype. To address this issue, we isolated two additional transgenic cell lines, one each expressing the WT and PEST-modified alleles of DNA ligase III. Analysis revealed that the levels of DNA ligase activity present in nuclear extracts prepared from the two cell lines were not appreciably different from each other (data not shown). These cell lines were then tested for their relative sensitivity to the DNA-damaging agents 9AC and IR. As Table I reveals, both of the cell lines expressing the PEST-modified DNA ligase III allele (referred to as MOD\textsubscript{A} and MOD\textsubscript{B}) are appreciably more resistant to the cytotoxic effects of these two agents than are the two cell lines expressing the WT DNA ligase allele (referred to as WT\textsubscript{A} and WT\textsubscript{B}). Interestingly, we observed that pretreatment with calpeptin provided equal levels of protection from the cytotoxic effects of these agents to cells, irrespective of whether they expressed the PEST-modified or WT DNA ligase III alleles (data not shown).

**DISCUSSION**

The results presented herein provide compelling evidence that DNA ligase III is a physiologically relevant substrate of the calcium-dependent cysteine protease calpain. Calpain binds to the N-terminal portion of the DNA ligase III protein wherein is located a PEST sequence. A modified form of the DNA ligase III protein in which the PEST sequence has been modified does not interact with calpain in the yeast two-hybrid system. In contrast to the WT DNA ligase III protein, this modified ligase protein is resistant to in vitro proteolysis by purified calpain. Exposure of human HT1080 cells to either ionizing radiation or the topoisomerase I inhibitor camptothecin resulted in DNA ligase III degradation. Pretreatment of these cells with the cell-permeant calpain inhibitor calpeptin simultaneously blocked DNA ligase III degradation and protected the cells from death induced by these agents. We have also shown that expression of the PEST-modified allele of DNA ligase III protects cells from IR- and camptothecin-induced cell death.

The finding that protease-resistant versions of both DNA ligase III and Rad51 (40) protect cells from death induced by IR indicates that their inactivation is an essential feature of programmed cell death. These findings indicate that DNA repair enzymes play a fundamental role in the process of programmed cell death.
cell death. Because overexpression of protease-sensitive versions of Rad51 and DNA ligase III provides less protection than does expression of protease-resistant versions, it is their ability to resist proteolysis, rather than their DNA repair activity per se, that is responsible for the protective effects of the latter enzymes.

Based on these findings, the following model can be proposed. Following exposure to high levels of IR or other DNA-damaging agents, such as topoisomerase inhibitors, a cell death signal is propagated. Although it is tempting to speculate that the trigger for this is the detection of a threshold level of DNA lesions, this is not a central feature of the model. One of the consequences of the cell death signal is the proteolysis of DNA ligase III by calpain. In cells expressing WT DNA ligase III, proteolysis of this enzyme would result in diminished cellular DNA repair capacity. As a consequence of the reduced repair capacity, the DNA lesions created by the ionizing radiation would persist, causing the cell death signal to continue unabated, ultimately resulting in cell death. In contrast, these activated proteases fail to degrade the protease-resistant form of DNA ligase III. In these cells, the DNA damage would be repaired, and the cell death signal is terminated, thus sparing the cells. Here, the critical factor determining whether cells live or die is the length of time that unrepaired DNA lesions persist in the cells. Alternately, one can propose that nucleases activated in response to DNA damage create additional genomic damage. This would create an amplification loop that propagates the cell death signal, leading to cell death. However, cells expressing a protease-resistant form of DNA ligase III would be able to repair the induced DNA damage, thereby dampening the amplification process and enhancing cell survival.

This model suggests that in addition to their structural role in restoring the integrity of the nuclear genome, DNA repair enzymes such as Rad51 and DNA ligase III play an important role in fine-tuning the trigger point of the cell death cascade. This would imply that relatively small differences in the levels of DNA repair activity that exist among different cells may have a dramatic effect on the ability of these cells to survive following exposure to DNA-damaging agents. This in turn would have a significant implication regarding the efficacy of cancer chemotherapy regimens in different patients.

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