A Role of the Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent Endonuclease in Apoptosis and Its Inhibition by Poly(ADP-ribose) Polymerase*

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Apoptosis is characterized by various cell morphological and biochemical features, one of which is the internucleosomal degradation of genomic DNA. The role of the human chromatin-bound Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-dependent endonuclease (CME) DNAS1L3 and its inhibition by poly(ADP-ribosyl)ation in the DNA degradation that accompanies apoptosis was investigated. The nuclear localization of this endonuclease is the unique feature that distinguishes it from other suggested apoptotic nucleases. Purified recombinant DNAS1L3 was shown to cleave nucleic acid into both high molecular weight and oligonucleosomal fragments in vitro. Furthermore, exposure of mouse skin fibroblasts expressing DNAS1L3 to inducers of apoptosis resulted in oligonucleosomal DNA fragmentation, an effect not observed in cells not expressing this CME, as well as in a decrease in cell viability greater than that apparent in the control cells. Recombinant DNAS1L3 was modified by recombinant human poly(ADP-ribose) polymerase (PARP) in vitro, resulting in a loss of nuclease activity. The DNAS1L3 protein also underwent poly(ADP-ribosyl)ation in transfected mouse skin fibroblasts in response to inducers of apoptosis. The cleavage and inactivation of PARP by a caspase-3-like enzyme late in apoptosis were associated with a decrease in the extent of DNAS1L3 poly(ADP-ribosyl)ation, which likely releases DNAS1L3 from inhibition and allows it to catalyze the degradation of genomic DNA.

Apoptosis, or programmed cell death, is an evolutionarily conserved process that is important in normal development, physiological homeostasis, and certain pathological conditions. It is mediated by a variety of intracellular enzymes, among which are endonucleases that catalyze the internucleosomal fragmentation of DNA, which is one of the hallmarks of apoptotic death (1, 2). Candidates for such endonucleases include the caspase-activated enzymes DFF40 (or CAD) (3–8) and NUC70 (9), divalent cation-dependent neutral (1, 2) or acidic (10, 11) endonucleases, leukemia-associated endo-exonucleases (12), and Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-dependent endonucleases (CMEs) (13–18).

CMEs introduce double strand breaks and single strand nicks into DNA, generating fragments with 5’-phosphate and 3’-hydroxyl termini, a mode of DNA fragmentation consistent with the products of chromatin degradation in apoptotic cells (19–21). CME activity is increased by a variety of stimuli that induce apoptosis (17, 21), and treatments that prevent apoptosis also prevent the induction of CME activity. A role for CMEs in apoptosis has also been supported by studies demonstrating the activation of DNA fragmentation by Ca\textsuperscript{2+} chelators or Zn\textsuperscript{2+} (13–15, 22).

One of earliest nuclear events in apoptosis is the poly(ADP-ribosyl)ation of various proteins by poly(ADP-ribose) polymerase (PARP), an enzyme that is activated by the presence of DNA strand breaks (23, 24). PARP catalyzes the modification of histones, topoisomerases I and II, SV40 large T antigen, DNA polymerase α, proliferating cell nuclear antigen, and various other DNA-binding proteins (25, 26). Indeed, we have previously shown that an early and transient burst of poly(ADP-ribosyl)ation of nuclear proteins, prior to the commitment to cell death, is required for apoptosis (23). Our observations suggested that subsequent cleavage of PARP by a caspase-3-like protease releases certain nuclear proteins from poly(ADP-ribosyl)ation-induced inhibition and thereby allows them to mediate DNA fragmentation and cell death (23). The activity of chromatin-bound CMEs purified from rat liver or thymus has been shown to be inhibited by poly(ADP-ribosyl)ation (27–31), however, until this time, a particular nuclease of this type had not been identified from human cells.

We previously identified the human homolog, DNAS1L3, of bovine and rat chromatin-bound CMEs. The nuclear localization of DNAS1L3 is the unique feature of this enzyme that distinguishes it from other suggested apoptotic nucleases (32). Our data indicated that this nuclease cleaves DNA into both oligonucleosomal and high molecular weight fragments (32). We now provide additional evidence that DNAS1L3 is regulated by PARP and that it may be responsible for apoptotic DNA degradation. The results also demonstrate that the activation of DNAS1L3 increases rates of cell death during apoptosis.

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¶ The abbreviations used are: CME, Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-dependent endonuclease; PARP, poly(ADP-ribose) polymerase; TNF-α, tumor necrosis factor-α; TAFE, transverse alternating-field electrophoresis; RT-PCR, reverse transcription and polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; AMC, aminomethylcoumarin; 3-AB, 3-aminobenzamide; PAR, poly(ADP-ribose); Pipes, 1,4-piperazinediethanesulfonic acid; kb, kilobase(s).
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**EXPERIMENTAL PROCEDURES**

Cell Culture—Mouse fibroblasts, immortalized by a standard 3T3 protocol, were kindly provided by Z. Q. Wang (International Agency for Research on Cancer, Lyon, France). Cells were grown under a humidified atmosphere of 5% CO_2 in air at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). They were maintained in the logarithmic phase of growth by passage every 2–3 days.

DNAS1L3 cDNA was aligned to the coding region for six histidine residues followed by the FLAG epitope and cloned in pcDNA3.1 mammalian expression vector (Invitrogen). Addition of the His-FLAG tag was necessary for analysis of protein expression and also for purification of the recombinant DNAS1L3, because antibodies against the native enzyme were not available. Cells were transfected with the use of the HiPerTrans 100 reagent (Promega) and selected by culture for 2–3 weeks in the presence of G418 and then pooled. Staurosporin, tumor necrosis factor-α (TNF-α), and antibodies to mouse Fas receptor were obtained from Sigma, Roche Molecular Biochemicals, and Kamiya Biomedical, respectively.

Production of Recombinant Proteins—Recombinant human PARP was purified essentially as described (33). A pET21a(+) expression vector (Novagen) containing the coding region for DNAS1L3 was used for the production of recombinant nuclease; because the 20 NH2-terminal residues followed by the FLAG epitope and cloned in pcDNA3.1 mammalian expression vector (Invitrogen). Addition of the His-FLAG tag was necessary for analysis of protein expression and also for purification of the recombinant DNAS1L3, because antibodies against the native enzyme were not available. Cells were transfected with the use of the HiPerTrans 100 reagent (Promega) and selected by culture for 2–3 weeks in the presence of G418 and then pooled. Staurosporin, tumor necrosis factor-α (TNF-α), and antibodies to mouse Fas receptor were obtained from Sigma, Roche Molecular Biochemicals, and Kamiya Biomedical, respectively.

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were analyzed by SDS-PAGE through a 12.5% gel. Gels were dried and sample buffer and heating at 95 °C for 10 min, after which samples were added. The reaction was terminated by the addition of an equal volume of SDS and linear forms of DNA generated from the supercoiled plasmid DNA (Invitrogen) in the presence of optimal conditions (2.5 mM MgCl₂ and 5 mM MgCl₂) and are means of triplicates from an experiment that was repeated three times with similar results.

RESULTS

Cation Dependence of DNA Cleavage by DNAS1L3—The results of our previous study (32) suggested that DNAS1L3 mediates Ca²⁺- and Mg²⁺-dependent fragmentation of DNA both in vitro and in vivo. We have now examined the cation dependence of DNAS1L3 directly with the use of the purified recombinant enzyme. The purified protein required both Ca²⁺ and Mg²⁺ for maximal activity; the optimal concentrations of these cations were 2.5–5 mM (Fig. 1A) and 5 mM (Fig. 1B), respectively, values similar to those determined for CME activity in rat liver nuclei (32). The activity of DNAS1L3 was also supported by Mn²⁺ (Fig. 1C), with 20 mM Mn²⁺ yielding ~80% of the nuclease activity apparent in the presence of optimal concentrations of Ca²⁺ and Mg²⁺; this concentration of Mn²⁺ is ~10 times that shown to be optimal for the rat homolog (DNase γ) of DNAS1L3 (39). The activity of DNAS1L3 in the presence of Ca²⁺ and Mg²⁺, like that of DNase γ (39), was inhibited by Zr²⁺, with a median effective concentration of ~45 μM (Fig. 1D).

Characterization of the Nature of DNA Strand Cleavage by Recombinant DNAS1L3—To examine whether DNAS1L3 preferentially introduces single stranded nicks or double stranded breaks into DNA substrates, we incubated various amounts of the recombinant enzyme with supercoiled and linear forms of pCR2.1 plasmid DNA (Invitrogen) in the presence of optimal concentrations of Ca²⁺ and Mg²⁺. Agarose gel electrophoresis of the reaction products revealed that DNAS1L3 cleaves both forms of DNA in a dose-dependent manner (Fig. 2). However, at low concentrations (2–4 μg/ml), the recombinant enzyme preferentially introduced single strand nicks rather than double strand breaks, as reflected by the relative abundance of relaxed and linear forms of DNA generated from the supercoiled plasmid (Fig. 2A).

Cleavage of Nuclear DNA by Recombinant DNAS1L3—We previously analyzed Ca²⁺- and Mg²⁺-dependent cleavage of DNA in nuclei isolated from rat liver and cerebellum (32). Although liver nuclei exhibited such DNA cleavage, cerebellar nuclei did not. Consistent with the notion that DNase γ is responsible for the observed DNA degradation in the liver nuclei, transcripts encoding this enzyme were detected in rat liver but not in cerebellum. We investigated this conclusion further in the present study by incubating rat cerebellar nuclei with recombinant human DNAS1L3 in the absence or presence of Ca²⁺ and Mg²⁺ and then analyzing the generation of high molecular weight and oligonucleosomal DNA fragments. TAFE revealed the presence of small amounts of >1000-kb DNA fragments in cerebellar nuclei incubated without DNAS1L3 in the absence or presence of Ca²⁺ and Mg²⁺ (Fig. 3A). Although Mg²⁺ alone did not induce the production of high molecular weight DNA fragments in nuclei incubated in the presence of recombinant DNAS1L3, nuclei incubated with DNAS1L3 in the presence of 5 mM Mg²⁺ and 0.1 mM Ca²⁺ exhibited a marked increase in the amount of >1000-kb DNA fragments. A further increase in the Ca²⁺ concentration to 2.5 mM resulted in processing of the >1000-kb DNA molecules into ~50-kb fragments (Fig. 3A). Electrophoresis through 1.5% agarose gels revealed that DNAS1L3 cleaved DNA in cerebellar nuclei into oligonucleosomal fragments in a manner that was dependent on both enzyme dose as well as Ca²⁺ and Mg²⁺ (Fig. 3B). No such internucleosomal DNA fragmentation was apparent in the absence of added DNAS1L3. These results thus provide direct evidence that DNAS1L3 catalyzes the cleavage of DNA into both high molecular weight and oligonucleosomal fragments.

Effect of DNAS1L3 Expression on DNA Fragmentation during Apoptosis—We next examined the potential role of DNAS1L3 in DNA cleavage during apoptosis and its effect on cell viability in mouse skin fibroblasts stably transfected with DNAS1L3 cDNA (32). The expression vector encoded DNAS1L3 fused at its COOH terminus with six histidine residues to facilitate protein purification with Ni-NTA agarose. The presence of this COOH-terminal tag did not inhibit DNAS1L3 activity, given that the recombinant nuclease expressed in and purified from bacteria for our in vitro experi-

FIG. 1. Cation dependence of DNAS1L3 activity. The nuclease activity of recombinant DNAS1L3 was measured at 37 °C in the presence of either 5 mM MgCl₂ and the indicated concentrations of CaCl₂ (A); 2.5 mM CaCl₂ and the indicated concentrations of MgCl₂ (B); the indicated concentrations of MnCl₂ (in the absence of Mg²⁺ and Ca²⁺) (C); or 2.5 mM CaCl₂, 5 mM MgCl₂, and the indicated concentrations of ZnSO₄ (D). Data are expressed as a percentage of the nuclease activity apparent under optimal conditions (2.5 mM CaCl₂ and 5 mM MgCl₂) and are means of triplicates from an experiment that was repeated three times with similar results.

FIG. 2. Analysis of mode of DNA strand cleavage catalyzed by recombinant DNAS1L3. Circular (A) or linear (B) pCR2.1 DNA (1 μg) was incubated for 1 h at 37 °C in the presence of 5 mM MgCl₂, 2.5 mM CaCl₂, and the indicated concentrations of purified recombinant DNAS1L3 in a final volume of 15 μl. The DNA was then analyzed by electrophoresis through a 1.5% agarose gel and ethidium bromide staining. S, L, and R indicate supercoiled, linear, and relaxed forms of the plasmid, respectively. M demonstrates the 1-kb DNA ladder molecular weight DNA standard.
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FIG. 3. Effect of recombinant DNAS1L3 on Ca$$^{2+}$$- and Mg$$^{2+}$$-dependent DNA fragmentation in isolated rat cerebellar nuclei. The DNA cleavage activity of the indicated amounts of DNAS1L3 was analyzed by incubation with rat cerebellar nuclei for 15 min (A) or 1 h (B) at 37°C in the presence of the indicated concentrations of Mg$$^{2+}$$ and Ca$$^{2+}$$. The integrity of the DNA was then evaluated by TAE (A) or conventional agarose gel electrophoresis (B). The leftmost lane in B contains molecular size standards.

<table>
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<th>10</th>
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<td>0.2</td>
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Table: DNA degradation induced by DNAS1L3

<table>
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<th>0.5</th>
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<tbody>
<tr>
<td>DNAS1L3 (mg)</td>
<td>5</td>
<td>10</td>
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<td>20</td>
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The effect of poly(ADP-ribo)sylation on DNAS1L3 activity was assessed qualitatively with a DNA degradation assay. Thus, although the unmodified enzyme effectively degraded bacteriophage λ DNA in the presence of optimal concentrations of Ca$$^{2+}$$ and Mg$$^{2+}$$, poly(ADP-ribo)sylated DNAS1L3 showed no such activity (Fig. 6A). The inclusion of 3-AB in, or the omission of NAD or PARP from, the poly(ADP-ribo)sylate reaction mixture prevented the inhibition of DNAS1L3.

Effect of Poly(ADP-ribo)sylation on DNAS1L3 Activity during Apoptosis—The effect of poly(ADP-ribo)sylation on DNAS1L3 activity was also examined in mouse fibroblasts stably expressing histidine-tagged DNAS1L3. The generation of oligonucleosomal DNA fragments was first evident in DNAS1L3-expressing cells ∼9 h after exposure to TNF-α and cycloheximide, whereas DNA fragmentation was not detected in control fibroblasts at any time point examined (Fig. 7A). DNA degradation in the DNAS1L3-expressing fibroblasts was accompanied by an increase in caspase-3-like activity; this activity was maximal 9 h after exposure to TNF-α and cycloheximide and decreased thereafter (Fig. 7B). Similar changes in caspase-3-like activity were observed in control cells treated with these agents (data not shown). Immunoblot analysis revealed that cleavage of PARP to yield an apoptosis-specific 89-kDa fragment was first apparent in the DNAS1L3-expressing (Fig. 7C) or control (data not shown) cells 3 h after exposure to TNF-α and cycloheximide; after 12 h of incubation, almost all of the 113-kDa PARP protein had been cleaved and inactivated, presumably as a result of the increase in caspase-3-like activity.

Immunoblot analysis with antibodies to poly(ADP-ribose) (PAR) revealed that the extent of poly(ADP-ribo)sylation of nuclear proteins increased during exposure of DNAS1L3-expressing cells to TNF-α and cycloheximide (Fig. 7D); this effect was maximal at 6 h, a time at which cells remained viable, and had declined markedly by 12 h, concomitant with the increases in caspase-3-like activity, cleavage of PARP, and DNA fragmentation. In mock-transfected fibroblasts similar changes in PAR synthesis have been observed during a course of apoptosis (data not shown).

Poly(ADP-ribo)sylation of nuclear proteins in response to DNA damage is transient and restricted to those proteins associated with PARP adjacent to DNA strand breaks (40). Although the bovine homolog of human DNAS1L3 was identified as a chromatin-bound enzyme (31, 32), our immunocytochemical data suggest that DNAS1L3 is localized mostly to the perinuclear region (not shown). Detection of poly(ADP-ribo)sylation of DNAS1L3, a reaction that occurs in the nucleus, might

Combiant human PARP in the presence of a low concentration of [32P]NAD and high molecular weight DNA resulted in marked poly(ADP-ribo)ylation of both proteins (Fig. 6A). This effect was blocked in the presence of the PARP inhibitor 3-aminobenzamide (3-AB). In the absence of DNAS1L3, automodification of PARP was not detected; however, when high molecular weight DNA in the reaction mixture was replaced with activated DNA, poly(ADP-ribo)sylation of PARP was apparent even in the absence of DNAS1L3. These results showed that DNAS1L3 activates PARP by introducing breaks into DNA strands, and that PARP, in turn, catalyzes the post-translational modification of the nuclease. The effect of the increasing NAD concentration of the reaction mixture from 1.3 μM to 3 mM on the length of the ADP-ribose chains attached to DNAS1L3 was investigated by performing the reaction with nonradioactive NAD. Under these conditions, the positions of both DNAS1L3 and PARP were shifted toward the top of the gel, reflecting the presence of long chains of ADP-ribose attached to these proteins (Fig. 6A).

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therefore be expected to be difficult in intact cells, because only a small proportion of this protein normally enters the cell nucleus. To investigate whether DNAS1L3 undergoes poly(ADP-ribosyl)ation during apoptosis, we therefore isolated the histidine-tagged nuclease from extracts of DNAS1L3-expressing mouse fibroblasts with the use of Ni-NTA magnetic agarose beads (Qiagen). The purified protein was then subjected to immunoblot analysis with antibodies to PAR. The extent of poly(ADP-ribosyl)ation of DNAS1L3 was low under normal culture conditions, was markedly increased 6 h after exposure of cells to TNF-α and cycloheximide, and had returned to control values after 12 h (Fig. 7E).

DISCUSSION

Early studies showing that liver nuclei contain endonuclease activity responsible for specific degradation of DNA implicated CMEs in internucleosomal DNA fragmentation (41, 42), and subsequent studies established a link between intracellular Ca²⁺ and induction of apoptosis (2, 43, 44). Previous observations also indicated that chromatin-bound CMEs from various tissues are poly(ADP-ribosyl)ated and thereby inhibited by PARP (27–32). PARP has been shown to be important in various cellular models of apoptosis, although the precise molecular mechanisms involved remain poorly understood (23–25, 45). PARP activation has been proposed to result in cell death by depletion of cellular NAD and ATP (46, 47). On the other hand, PARP is rapidly cleaved and inactivated by caspases (48), and this cleavage is thought to be a key apoptotic event (49, 50).

We have recently shown that human DNAS1L3 is necessary for Ca²⁺- and Mg²⁺-dependent internucleosomal DNA cleavage in specific cell types (32). With the use of recombinant DNAS1L3, we have now confirmed that DNAS1L3 requires both Ca²⁺ and Mg²⁺ for maximal activity, with optimal concentrations of 2.5–5 mM for Ca²⁺ and 5 mM for Mg²⁺, consistent with the values we previously obtained for DNA fragmentation in isolated rat liver nuclei (32). We also showed that, like other DNase I-related nucleases (39, 51), human DNAS1L3 is activated by Mn²⁺ and inhibited by Zn²⁺. Kinetic analysis of plasmid DNA cleavage by recombinant DNAS1L3 has revealed that the nuclease preferentially introduces nicks into one strand of double stranded DNA rather than catalyzing the cleavage of both strands; however, at higher concentrations, the enzyme also mediates double strand scission.

We previously showed that rat liver nuclei, but not nuclei isolated from rat cerebellum, exhibit fragmentation of DNA into high molecular weight and oligonucleosomal fragments on incubation in the presence of Ca²⁺ and Mg²⁺ (32). Furthermore, we detected DNase γ mRNA in rat liver but not in rat cerebellum (32). We thus hypothesized that DNase γ is responsible for both types of DNA cleavage stimulated by Ca²⁺ and Mg²⁺ in rat liver nuclei. These results indicate that preferential appearance of oligonucleosomal or high molecular weight DNA fragments may depend on levels of CME activity.

Mouse fibroblasts expressing human DNAS1L3, but not con-
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control cells, exhibited extensive internucleosomal DNA fragmentation in response to inducers of apoptosis. Although internucleosomal DNA fragmentation is a biochemical marker of apoptosis, its precise role in cell death is unclear. Expression of DNAS1L3 in mouse fibroblasts markedly increased the incidence of apoptosis induced by various treatments, consistent with the results of previous studies (13–15, 22), suggesting that Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-dependent internucleosomal DNA fragmentation during apoptosis in mouse fibroblasts. Cells transfected with a DNAS1L3 expression plasmid were incubated for various times in the presence of TNF-α (10 ng/ml) and 1 μM cycloheximide, after which internucleosomal DNA fragmentation was analyzed by electrophoresis through a 2% agarose gel (A); caspase-3-like activity was assayed fluorometrically (data are expressed as a percentage of the value for time zero and are from a representative experiment) (B); cleavage of PARP (C) and poly(ADP-ribosylation) of nuclear proteins (D) were monitored by immunoblot analysis with antibodies to PARP and to PAR, respectively; and the recombinant histidine-tagged DNAS1L3 protein was purified from the transfected cells and subjected to immunoblot analysis with antibodies to PAR (E). Data for control (C) cells transfected with the empty vector are also shown in A and E.

Our results thus suggest a model for apoptosis (Fig. 8) in which the activation of DNAS1L3 by an increase in the intracellular concentration of Ca\textsuperscript{2+} results in the introduction of strand breaks into genomic DNA and the consequent activation of PARP. Poly(ADP-ribosylation) of DNAS1L3 by PARP, in turn, results in inhibition of nuclease activity. Subsequent cleavage and inactivation of PARP by caspases prevents further poly(ADP-ribosylation) of nuclear proteins, thereby allowing the activity of PAR glycohydrolase to remove polymer from these proteins and thereby release DNAS1L3 from inhibition. The activated nuclease may then catalyze the internucleosomal DNA fragmentation characteristic of the later stages of apoptosis. Currently, experiments initiated utilizing caspase-3-deficient cells strengthen our hypothesis, because established peptide inhibitors of caspase 3 have only limited specificity.

Automodification of PARP, followed by the subsequent removal of the polymer during a cycling mechanism of protein activity and binding to DNA strands breaks during DNA replication and repair, has been verified in several studies, includ-
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Fig. 8. Model for the role of DNAISIL3 in DNA fragmentation during apoptosis. The apoptotic stimulus may result in initial DNA damage directly or through Ca\(^{2+}\)-induced activation of DNAISIL3. See text for further details.

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REFERENCES

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