

Regulation of DNAS1L3 Endonuclease Activity by Poly(ADP-ribose)ation during Etoposide-induced Apoptosis

ROLE OF POLY(ADP-RIBOSE) POLYMERASE-1 CLEAVAGE IN ENDONUCLEASE ACTIVATION*

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Several endonucleases are implicated in the internucleosomal DNA fragmentation associated with apoptosis. The human Ca^{2+} - and Mg^{2+} -dependent endonuclease DNAS1L3 is inhibited by poly(ADP-ribose)ation *in vitro*, and its activation during apoptosis shows a time course similar to that of the cleavage of poly(ADP-ribose) polymerase-1 (PARP-1). The role of the cleavage and consequent inactivation of PARP-1 by caspase-3 in the activation of DNAS1L3 has now been investigated further both *in vitro* and *in vivo*. In an *in vitro* system based on purified recombinant proteins and NAD, caspase-3 prevented the inhibition of DNAS1L3 endonuclease activity by wild-type PARP-1 but not that induced by a caspase-3-resistant PARP-1 mutant. The induction by etoposide of apoptosis in human osteosarcoma cells (which were shown not to express endogenous DNAS1L3) was accompanied by internucleosomal DNA fragmentation only after transfection of the cells with a plasmid encoding DNAS1L3. This DNA fragmentation in etoposide-treated cells was blocked by 1,2-bis(2-amino-phenoxy)-ethane-*N,N,N',N'*-tetraacetic acid, an inhibitor of intracellular Ca^{2+} release. Expression of the endonuclease subunit of DNA fragmentation factor (DFF40) and cleavage of its inhibitor, DFF45, were not sufficient to cause internucleosomal DNA fragmentation in osteosarcoma cells during etoposide-induced apoptosis. Coexpression of caspase-3-resistant PARP-1 mutant with DNAS1L3 in osteosarcoma cells blocked etoposide-induced internucleosomal DNA fragmentation and resulted in persistent poly(ADP-ribose)ation of DNAS1L3; it did not, however, prevent the activation of caspase-3 and the consequent cleavage of endogenous PARP-1. These results indicate that PARP-1 cleavage during apoptosis is not simply required to prevent excessive depletion of NAD and ATP but is also necessary to release DNAS1L3 from poly(ADP-ribose)ation-mediated inhibition.

Apoptosis plays important roles in immunity, development, and homeostasis as well as in the response to cell injury. This process of programmed cell death is characterized by marked changes in cell morphology, including chromatin condensation, membrane blebbing, nuclear breakdown, and the appearance of membrane-associated apoptotic bodies, as well as by internucleosomal DNA fragmentation and the cleavage of many housekeeping proteins such as poly(ADP-ribose) polymerase-1 (PARP-1)¹ and lamins. Apoptosis is triggered by various agents, including endogenous cytokines as well as therapeutic and cytotoxic drugs, and its initiation and execution are mediated by activation of members of the caspase family of aspartate-specific cysteine proteases.

Fragmentation of DNA is thought to be an important step in disposal of the genome in cells undergoing apoptosis. Defective DNA fragmentation has been associated with an increased resistance of cells to apoptosis (1–3). The mechanism of DNA fragmentation in apoptotic cells is poorly understood, although several endonucleases have been implicated in this process (4, 5). The various candidate endonucleases identified to date differ in characteristics such as Ca^{2+} and Mg^{2+} dependence, optimum pH, tissue distribution, and requirement for caspase-3 (5–8). DNA fragmentation factor (DFF), also known as caspase-activated DNase (CAD), has been suggested to play a major role in DNA fragmentation during apoptosis (5, 9–11). DFF is composed of two subunits of 40 and 45 kDa, termed DFF40 (CAD) and DFF45 (ICAD), respectively (3, 9–11). The endonuclease activity of this enzyme, which is intrinsic to DFF40, is induced on cleavage of DFF45 by caspase-3. We have recently cloned and characterized the human homolog (12, 13), termed DNAS1L3, of a bovine chromatin-bound, Ca^{2+} - and Mg^{2+} -dependent endonuclease (14) that is also thought to contribute to DNA fragmentation during apoptosis. The activity of DNAS1L3 (13), like that of the bovine endonuclease (14, 15), is inhibited by poly(ADP-ribose)ation.

PARP-1 is thought to be one of the earliest targets for cleavage by caspase-3-like proteases during apoptosis. Its cleavage into 89- and 24-kDa fragments renders PARP-1 inactive. With the use of a caspase-3-resistant PARP-1 mutant, we have recently shown that cleavage of PARP-1 plays an important role in the normal progression of apoptosis and that interference with PARP-1 cleavage increases the rate of apoptotic cell death as a result of excessive depletion of the PARP-1 substrate NAD

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¹ The abbreviations used are: PARP-1, PAR polymerase-1; PAR, poly(ADP-ribose); mut-PARP-1, caspase-3-resistant PARP-1 mutant; DFF, DNA fragmentation factor; TNF, tumor necrosis factor; RT, reverse transcription; AMC, aminomethylcoumarin; BAPTA, 1,2-bis(2-amino-phenoxy)-ethane-*N,N,N',N'*-tetraacetic acid.

(16). Hecceg and Wang (17) showed that expression of a similar caspase-3-resistant mutant of PARP-1 switched the mode of cell death induced by tumor necrosis factor (TNF) from apoptosis to necrosis. The cleavage of PARP-1 into inactive peptides on exposure of cells to inducers of apoptosis is thus thought to avoid excessive depletion of energy reserves and a switch to necrosis. Cleavage and inactivation of PARP-1 by caspase-3-like proteases during apoptosis also may be necessary for activation of the endonuclease DNAS1L3.

In a continuation of our investigations into the contribution of PARP-1 to mid and late stages of apoptosis, we have now examined the role of PARP-1 activity and cleavage of this enzyme by caspases in DNAS1L3-mediated DNA fragmentation, both with the use of an *in vitro* assay and in human osteosarcoma cells treated with the chemotherapeutic and proapoptotic drug etoposide. Our results demonstrate, for the first time, that PARP-1 and its cleavage by caspases play a direct role in DNAS1L3-mediated internucleosomal DNA fragmentation in intact cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Induction of Apoptosis—Human osteosarcoma cells (143.98.2; ATCC CRL 11226) (18, 19) and U-937 human monocytic cells (ATCC CRL-1593.2) (20) were maintained in Dulbecco's modified Eagle's medium and RPMI 1640 medium, respectively, each supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). Osteosarcoma cells were transfected with vectors encoding human DNAS1L3 (12) or a caspase-3-resistant PARP-1 mutant (mut-PARP-1) (16) with the use of Mirus TransIT-100 reagent (Panvera, Madison, WI). Both DNAS1L3 and mut-PARP-1 cDNAs were positioned immediately downstream of the coding sequence for six histidine residues, followed by the FLAG epitope, and were cloned in pcDNA3.1 and pCR3.1 mammalian expression vectors (Invitrogen, Carlsbad, CA), respectively. The addition of the His₆-FLAG tag was necessary for analysis of protein expression as well as for precipitation of the expressed proteins; antibodies to native DNAS1L3 are not available. Transfected osteosarcoma clones were isolated by selection with G418 or hygromycin (Sigma), or both, and were tested for expression of DNAS1L3 or mut-PARP-1 by immunoblot analysis with antibodies to the FLAG epitope as described below. Apoptosis was induced by exposing cells to 70 µM etoposide (Sigma) for 12 or 24 h at 37 °C.

Purification of Recombinant Proteins and *in Vitro* DNA Fragmentation Assay—Recombinant human wild-type PARP-1 and catalytically inactive PARP-1 were purified essentially as described (21). The mut-PARP-1 protein was partially purified from extracts of transfected osteosarcoma cells by precipitation with nickel-nitrilotriacetic acid magnetic beads (Qiagen, Valencia, CA). For assay of DNA fragmentation *in vitro*, the recombinant proteins DNAS1L3 (1 µg) (Alexis Biochemicals, San Diego, CA), wild-type PARP-1 (0.2 µg), inactive mutant PARP-1 (0.2 µg), the caspase-3-resistant mut-PARP-1 (1 µg), and caspase-3 (0.1 µg) (Alexis Biochemicals, San Diego, CA) were incubated for 60 min at 37 °C in a final volume of 30 µl of a solution containing 25 mM Tris-HCl (pH 7.4), 150 mM KCl, 5 mM MgCl₂, 2.5 mM CaCl₂, 1 µg of genomic DNA, and 0.5 mM NAD. The integrity of the DNA was then analyzed by electrophoresis through a 1.5% agarose gel and staining with ethidium bromide.

Assay of PARP-1 Cleavage *in Vitro*—Plasmids encoding wild-type PARP-1 or mut-PARP-1 tagged with His₆-FLAG were used to synthesize [³⁵S]methionine-labeled proteins by coupled T7 RNA polymerase-mediated transcription and translation in a reticulocyte lysate (Promega, Madison, WI). Lysates (1 µl) containing either recombinant protein were incubated for 30 min at 37 °C in the absence or presence of 10 ng of caspase-3. The reaction was terminated by the addition of SDS sample buffer, and the products were analyzed by SDS-PAGE and autoradiography.

RT-PCR Analysis—Total RNA was isolated from osteosarcoma or U-937 cells with the use of an Rneasy Mini kit (Qiagen, Valencia, CA), and 10-µg portions were subjected to reverse transcription (RT) with HotStarTaq (Qiagen, Valencia, CA). One-tenth of the resulting cDNA was amplified by PCR with the following primers (sense and antisense, respectively): DNAS1L3, 5'-GTTCCAATCTCCCCACACTG-3' and 5'-GTCTCTAAGCACAATCCTG-3'; human glyceraldehyde-3-phosphate dehydrogenase, 5'-TCTGCCCTCTGCTGATGC-3' and 5'-CCACACCCTGTTGCTGTAG-3'. Amplification was performed for 30 cycles

of denaturation for 30 s at 94 °C, annealing for 15 s at 55 °C, and primer extension for 60 s at 72 °C, and the resulting products were analyzed by electrophoresis through 2% agarose gels and staining with ethidium bromide. The identity of specific PCR products was confirmed by DNA sequencing.

Analysis of DNA Fragmentation—DNA was isolated from cells as described previously (16) and subjected to electrophoresis through 1.5% agarose gels in Tris borate/EDTA buffer. The gels were stained with ethidium bromide.

Immunoblot Analysis—Cells were washed with ice-cold phosphate-buffered saline and then lysed as described (16, 22). A portion (30 µg of protein) of each lysate was fractionated by SDS-PAGE on a 4–20% gradient gel. Alternatively, recombinant DNAS1L3 was precipitated from cell lysates (250 µg of protein) with nickel-nitrilotriacetic acid magnetic beads (Qiagen, Valencia, CA), and the precipitate was subjected to SDS-PAGE. Separated proteins were transferred to a nitrocellulose filter. The filters were stained with Ponceau S to confirm equal loading and transfer of samples and were then probed with antibodies to FLAG (Santa Cruz Biotechnology, Santa Cruz, CA), to PARP-1 (PharMingen, San Diego, CA), to the 89-kDa cleavage fragment of PARP-1 (Promega, Madison, WI), to poly(ADP-ribose) (PAR) (Alexis Biochemicals, San Diego, CA), and to DFF-45 or DFF-40 (kindly provided by Dr. X. Wang, University of Texas). Immune complexes were detected with appropriate secondary antibodies and chemiluminescence reagents (Pierce).

Assay of Caspase-3-like Activity—Caspase-3-like activity was measured essentially as described (16, 22). In brief, cell extracts (25 µg of protein) were incubated for 30 min at 37 °C with 40 µM DEVD-AMC peptide substrate in a total volume of 200 µl. The fluorescence of free aminomethylcoumarin (AMC), generated as a result of cleavage of the aspartate-AMC bond, was monitored continuously for 30 min with a CytoFluor 4000 fluorometer at excitation and emission wavelengths of 360 and 460 nm, respectively. The emission from each well was plotted against time, and linear regression analysis of the initial velocity (slope) for each curve yielded the activity.

RESULTS

Effect of PARP-1 Cleavage by Caspase-3 on DNAS1L3-mediated DNA Fragmentation *in Vitro*—With the use of an *in vitro* system, we have shown previously (13) that inactivation of PARP-1 by 3-aminobenzamide blocks the inhibition by PARP-1 of DNAS1L3-mediated DNA degradation. We have also demonstrated previously that DNAS1L3-mediated internucleosomal DNA fragmentation in transfected fibroblasts exposed to TNF and cycloheximide occurs immediately after PARP-1 cleavage, suggesting that the cleavage and consequent inactivation of PARP-1 might be required for the induction of DNAS1L3 endonuclease activity in cells. With the use of our *in vitro* system, we have now examined the effect of inactivation of PARP-1 by caspase-3 on DNAS1L3-mediated DNA fragmentation.

Recombinant human DNAS1L3 was incubated with recombinant human PARP-1 in the presence of NAD and genomic DNA and in the absence or presence of recombinant human caspase-3. As shown previously, DNAS1L3 mediated the complete degradation of genomic DNA (Fig. 1A), and this action was blocked by the addition of wild-type PARP-1. In contrast, a catalytically inactive mutant of PARP-1 had no effect on DNAS1L3-mediated DNA degradation, demonstrating that poly(ADP-ribose)ation catalyzed by PARP-1 is required for the inhibition of DNAS1L3 endonuclease activity. The presence of caspase-3 completely blocked the inhibition by PARP-1 of DNAS1L3 endonuclease activity, suggesting that PARP-1 cleavage by caspase-3 promotes the activation of this nuclease.

To confirm that the cleavage and consequent inactivation of PARP-1 by caspase-3 are required for DNAS1L3 endonuclease activity in our *in vitro* system, we examined the effect of a partially purified caspase-3-resistant mutant of PARP-1 (mut-PARP-1), in which the aspartate residue (Asp²¹⁴) at the caspase-3 cleavage site had been replaced with a glycine residue by site-directed mutagenesis. The catalytic activity and structural integrity of this mutant protein were unaffected

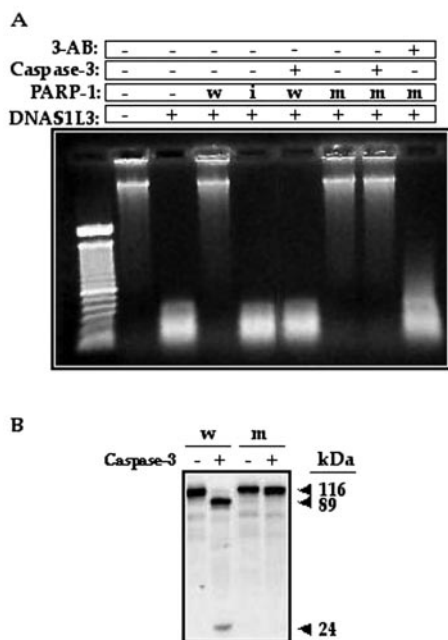


FIG. 1. Requirement of PARP-1 cleavage by caspase-3 for DNAS1L3-mediated DNA degradation *in vitro*. **A**, the indicated combinations of recombinant DNAS1L3 (added last), wild-type (*w*) PARP-1, catalytically inactive (*i*) PARP-1, caspase-3-resistant mutant (*m*) PARP-1, and caspase-3 were incubated in the absence or presence of 3 mM 3-aminobenzamide (3-AB) for 60 min at 37 °C in a reaction mixture containing genomic DNA and NAD. The integrity of DNA was then analyzed by agarose gel electrophoresis and ethidium bromide staining. The *leftmost* lane contains DNA size markers (1-kb intervals). **B**, recombinant wild-type PARP-1 (*w*) or the caspase-3-resistant mutant PARP-1 (*m*), each synthesized by *in vitro* transcription and translation in the presence of [³⁵S]methionine, was incubated in the absence or presence of recombinant caspase-3 for 30 min at 37 °C, after which cleavage products were detected by SDS-PAGE and autoradiography. The positions of the 116-kDa intact proteins and of the 89- and 24-kDa cleavage products are indicated on the *right*.

either by recombinant caspase-3 *in vitro* (Fig. 1B) (16) or when expressed in osteosarcoma cells induced to undergo apoptosis by staurosporine (16). The recombinant mut-PARP-1 completely blocked DNAS1L3 endonuclease activity in the absence or presence of caspase-3 (Fig. 1A). To demonstrate that inhibition of DNAS1L3 by mut-PARP-1 required the catalytic activity of the mutant, we examined the effect of 3-aminobenzamide. This drug prevented the inhibition of DNAS1L3 endonuclease activity by mut-PARP-1, confirming that PARP-1 activity is specifically required for the inhibition of DNAS1L3. These *in vitro* results thus indicate that the cleavage and consequent inactivation of PARP-1 by caspase-3 are necessary for the induction of DNAS1L3 endonuclease activity.

Etoposide-induced Internucleosomal DNA Fragmentation in Osteosarcoma Cells Expressing Recombinant DNAS1L3—During a search for a cell type with which to study the role of DNAS1L3 in DNA fragmentation during apoptosis, we found that human osteosarcoma cells (unlike U-937 human monocytes) do not express DNAS1L3, as revealed by RT-PCR analysis with primers specific for human DNAS1L3 cDNA (Fig. 2A). Furthermore, again unlike U-937 cells, the osteosarcoma cells do not undergo internucleosomal DNA fragmentation in response to the pro-apoptotic drug etoposide (Fig. 2B). We therefore transfected osteosarcoma cells with an expression vector that encodes DNAS1L3 fused to a His₆-FLAG tag. Immunoblot analysis with antibodies to the FLAG epitope revealed that the transfected cells expressed recombinant DNAS1L3 (Fig. 2C). Exposure of the transfected osteosarcoma cells to etoposide resulted in marked internucleosomal DNA fragmentation, and

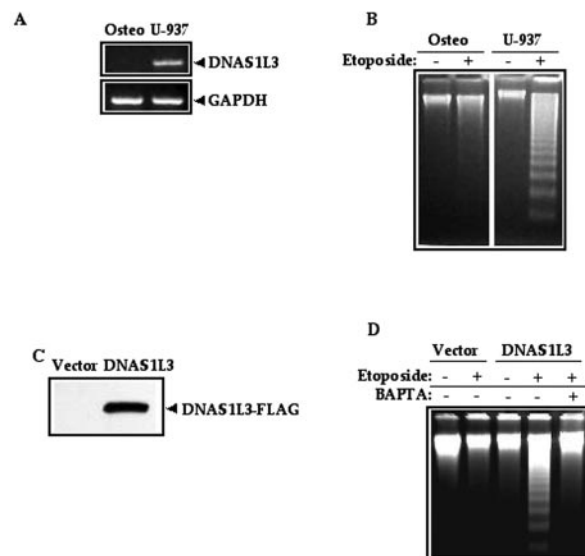


FIG. 2. Etoposide-induced internucleosomal DNA fragmentation in osteosarcoma cells expressing recombinant DNAS1L3. **A**, lack of DNAS1L3 expression in osteosarcoma cells. Total RNA isolated from human osteosarcoma cells or U-937 monocytes was subjected to RT-PCR with primers specific for DNAS1L3 or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNAs. The PCR products were analyzed by agarose gel electrophoresis and staining with ethidium bromide. **B**, absence of internucleosomal DNA fragmentation in osteosarcoma cells exposed to etoposide. Osteosarcoma or U-937 cells were incubated for 24 h in the absence or presence of 70 μM etoposide, after which genomic DNA was isolated and examined by agarose gel electrophoresis and ethidium bromide staining. **C**, expression of recombinant DNAS1L3 in osteosarcoma cells. Osteosarcoma cells were transfected either with an expression vector encoding DNAS1L3 tagged with the His₆-FLAG sequence or with the corresponding empty vector (*Vector*). Cell lysates were subsequently subjected to immunoblot analysis with antibodies to FLAG. **D**, effect of DNAS1L3 expression on DNA integrity in etoposide-treated osteosarcoma cells. Osteosarcoma cells transfected with the DNAS1L3 expression vector or the corresponding empty vector were incubated for 24 h in the absence or presence of 70 μM etoposide or 10 μM BAPTA, as indicated. Genomic DNA was then isolated from the cells and analyzed by agarose gel electrophoresis and ethidium bromide staining.

this effect was completely blocked by the presence in the incubation medium of a cell-permeable BAPTA, which inhibits intracellular Ca²⁺ release. These results demonstrated that DNAS1L3 mediates internucleosomal DNA fragmentation in the transfected osteosarcoma cells and that this activity is dependent on Ca²⁺, consistent with the results of our previous study (12) showing a requirement of Ca²⁺ for DNAS1L3 endonuclease activity, *in vitro*.

DFF40 Expression and DFF45 Cleavage Are Insufficient for Internucleosomal DNA Fragmentation in Osteosarcoma Cells during Etoposide-induced Apoptosis—Because DFF has been suggested to play a major role in DNA fragmentation during apoptosis (3, 5, 9–11), it was thus important to determine whether failure of osteosarcoma cells to undergo internucleosomal DNA fragmentation was associated with either an alteration in DFF40 and DFF45 protein expression or to a defect in DFF45 proteolytic processing after etoposide treatment. DFF40 and DFF45 protein expressions were, therefore, assessed in extracts from osteosarcoma cells and compared with those from U-937 cells by Western immunoblot analysis with antibodies to either human DFF40 or DFF45. Fig. 3A shows that both DFF40 and DFF45 protein levels were comparable with those detected in U-937 cells. We then examined the fate of DFF45 in extracts from cells that were treated with etoposide for 12 or 24 h. Fig. 3B shows that DFF45 was proteolytically cleaved into its typical apoptotic 32- and 11-kDa frag-

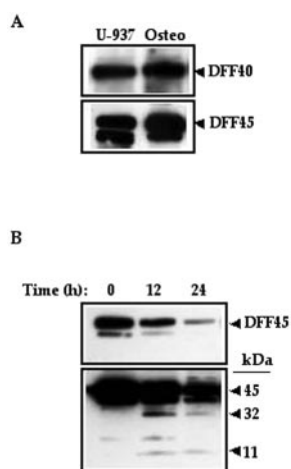


FIG. 3. DFF40 expression and DFF45 cleavage are insufficient for internucleosomal DNA fragmentation in osteosarcoma cells during etoposide-induced apoptosis. A, expression of DFF45 and DFF40 in osteosarcoma (*Osteo*) and U-937 cells. Cells were lysed and subjected to immunoblot analysis with antibodies to DFF40 (*top panel*) or to DFF45 (*bottom panel*). B, cleavage of DFF45 in osteosarcoma cells during etoposide-induced apoptosis. Osteosarcoma cells were incubated for 0, 12, or 24 h in the presence of 70 μM etoposide, after which cell lysates were prepared and were subjected to immunoblot analysis with antibodies to human DFF45; the *bottom panel* in B represents a longer exposure of the filter shown in the *top panel*. The positions of the 45-kDa intact protein and of the 32- and 11-kDa cleavage products are indicated on the *right*.

ments. Altogether, these results suggest that expression of DFF40 and cleavage of DFF45 are not sufficient to cause internucleosomal DNA fragmentation in osteosarcoma cells during etoposide-induced apoptosis.

Effect of mut-PARP-1 Expression on DNAS1L3-mediated DNA Fragmentation in Osteosarcoma Cells—To examine the role of PARP-1 cleavage in the induction of DNAS1L3 endonuclease activity in osteosarcoma cells, we transfected the cells with vectors encoding DNAS1L3, mut-PARP-1 (also fused to the His₆-FLAG sequence), or both of these proteins. Immunoblot analysis with antibodies to FLAG confirmed the expression of the recombinant proteins in the transfected cells (Fig. 4A). We next examined the effect of mut-PARP-1 expression on DNAS1L3-mediated internucleosomal DNA fragmentation by incubating transfected cells in the presence of etoposide for 12 or 24 h. Expression of mut-PARP-1 together with DNAS1L3 blocked etoposide-induced DNA fragmentation mediated by the recombinant endonuclease (Fig. 4B), presumably by maintaining it in the poly(ADP-ribosyl)ated (inhibited) state. Expression of mut-PARP-1 alone had no effect on the integrity of DNA in cells exposed (or not) to etoposide. These results suggest that cleavage of PARP-1 is necessary for DNAS1L3-mediated degradation of DNA and are consistent with the *in vitro* data shown in Fig. 1. The recombinant mut-PARP-1 protein remained intact during incubation of cells with etoposide (Fig. 4C).

Persistent Poly(ADP-ribosyl)ation of DNAS1L3 in Etoposide-treated Osteosarcoma Cells Expressing mut-PARP-1—To examine the relation between the endonuclease activity and the poly(ADP-ribosyl)ation state of DNAS1L3, we precipitated DNAS1L3 from etoposide-treated osteosarcoma cells expressing the endonuclease in the absence or presence of mut-PARP-1. The precipitates were then subjected to immunoblot analysis with antibodies to PAR. Cells expressing only DNAS1L3 exhibited a transient increase in the extent of poly(ADP-ribosyl)ation of this protein that was apparent after exposure to etoposide for 12 h but not after 24 h (Fig. 5). In contrast, coexpression of mut-PARP-1 was associated with a

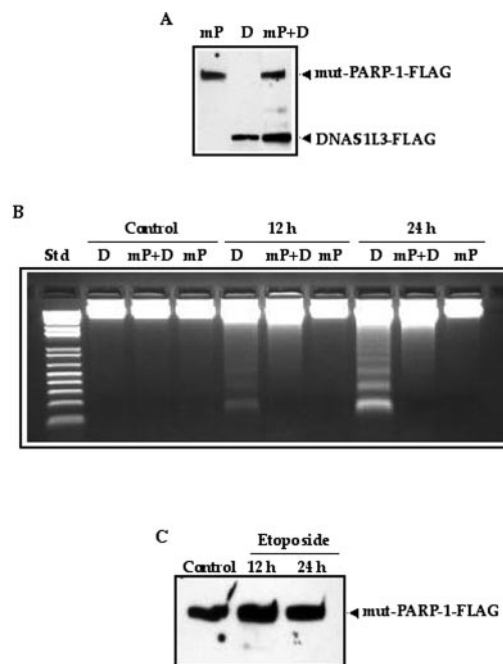


FIG. 4. Inhibition of DNAS1L3-mediated internucleosomal DNA fragmentation in osteosarcoma cells by expression of the caspase-3-resistant mut-PARP-1. A, expression of DNAS1L3 and mut-PARP-1 in osteosarcoma cells. Cells were transfected with vectors encoding either DNAS1L3 (*D*) or mut-PARP-1 (*mP*) each fused to the His₆-FLAG sequence, as indicated. Cell lysates were subsequently subjected to immunoblot analysis with antibodies to FLAG. B, effect of mut-PARP-1 expression on DNAS1L3-mediated DNA fragmentation in response to etoposide. Cells expressing recombinant DNAS1L3 (*D*), mut-PARP-1 (*mP*), or both proteins (*mP+D*) were incubated for 12 or 24 h in the presence of 70 μM etoposide or left untreated (*Control*), after which internucleosomal DNA fragmentation was analyzed by electrophoresis. The *leftmost lane* contains molecular size standards (*Std*) (1-kb intervals). C, fate of mut-PARP-1 in etoposide-treated osteosarcoma cells. Cells expressing both DNAS1L3 and mut-PARP-1 were incubated in the presence of 70 μM etoposide for 0, 12, or 24 h or left untreated (*Control*), after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to FLAG.

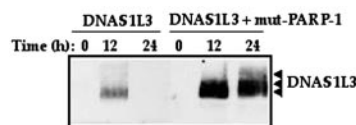


FIG. 5. Persistent poly(ADP-ribosyl)ation of DNAS1L3 induced by etoposide in cells expressing mut-PARP-1. Osteosarcoma cells expressing either DNAS1L3 or both mut-PARP-1 and DNAS1L3 were incubated for the indicated times with 70 μM etoposide, after which the recombinant His₆-FLAG-tagged proteins were precipitated and then subjected to immunoblot analysis with antibodies to PAR.

markedly greater increase in the extent of poly(ADP-ribosyl)ation of DNAS1L3 after incubation with etoposide for 12 h, and this level of modification was still apparent at 24 h. These results appear to correlate with the kinetics of DNA fragmentation in the transfected cells, and they suggest that hydrolysis of PAR moieties attached to DNAS1L3 by the action of PARP-1 is required for induction of the endonuclease activity of this protein in cells undergoing apoptosis.

Caspase-3-like Activity and Cleavage of Endogenous PARP-1 in Etoposide-treated Osteosarcoma Cells Expressing mut-PARP-1, DNAS1L3, or Both Proteins—To verify that the lack of internucleosomal DNA fragmentation in etoposide-treated osteosarcoma cells expressing both DNAS1L3 and mut-PARP-1 was not due to inhibition of the activation of other apoptotic factors, we measured caspase-3-like activity. Cells were incubated for 12 or 24 h in the presence of etoposide, after which cell

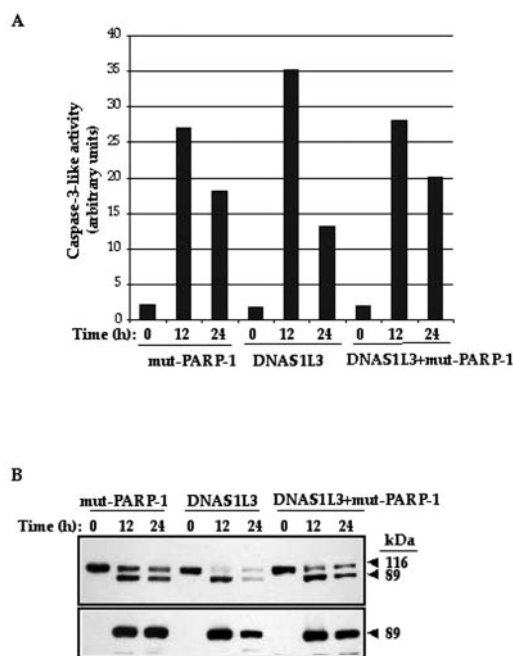


FIG. 6. Etoposide-induced activation of caspase-3 and cleavage of endogenous PARP-1 in osteosarcoma cells expressing mut-PARP-1 and DNAS1L3. Cells expressing mut-PARP-1, DNAS1L3, or both recombinant proteins were incubated for 0, 12, or 24 h in the presence of 70 μ M etoposide, after which cell lysates were prepared and were either assayed for caspase-3-like activity with the fluorogenic substrate DEVD-AMC (A) or subjected to immunoblot analysis (B); the latter analysis was performed with antibodies either to PARP-1 (upper panel) or to the 89-kDa cleavage fragment of PARP-1 (lower panel). Data in A are expressed in arbitrary units and are from a representative experiment.

extracts were prepared and assayed for caspase-3-like activity with the substrate DEVD-AMC. Etoposide induced a marked increase in caspase-3-like activity in osteosarcoma cells expressing mut-PARP-1, DNAS1L3, or both recombinant proteins (Fig. 6A), indicating that the failure of cells expressing both DNAS1L3 and mut-PARP-1 to undergo internucleosomal DNA fragmentation in response to etoposide was not due to a lack of caspase-3 activity.

The same cell extracts were also subjected to immunoblot analysis with antibodies to PARP-1, which recognize both the full-length protein and its 89-kDa cleavage product but not the His₆-FLAG-tagged recombinant mut-PARP-1 (16). Etoposide induced the cleavage of endogenous PARP-1 in each of the three types of transfected cells (Fig. 6B, upper panel). Similar results were obtained when the cleavage of endogenous PARP-1 was monitored with the use of antibodies that recognize only the 89-kDa cleavage fragment (Fig. 6B, lower panel).

The maximal level of caspase-3-like activity (Fig. 6A) and the extent of cleavage of endogenous PARP-1 (Fig. 6B) were higher in cells expressing only DNAS1L3 than in those expressing mut-PARP-1 alone or both mut-PARP-1 and DNAS1L3. These results are consistent with the higher sensitivity of the former cells to etoposide (data not shown).

DISCUSSION

PARP-1 is cleaved by caspase-3 or caspase-7 early during apoptosis in many cell lines and tissues. Several caspase substrates have been shown to play a direct positive or negative role in apoptosis. Among these substrates are pro-apoptotic proteins such as caspases themselves (23, 24) and Bid (25), whose cleavage results in activation and escalation of the apoptotic pathway, as well as anti-apoptotic proteins such as members of the Bcl-2 family (26) and the transcription factor NF- κ B

(27), which, if not cleaved, block the death program. Although the role of PARP-1 cleavage in apoptosis remains to be fully elucidated, substantial insight into this role has been gained during the past several years (3, 16, 17, 28, 29).

The cleavage of PARP-1 between Asp²¹⁴ and Gly²¹⁵ results in separation of the two zinc finger DNA-binding motifs in the NH₂-terminal region of the enzyme from the automodification and catalytic domains, thus preventing recruitment of the catalytic domain to sites of DNA damage (30, 31). This cleavage of PARP-1 has been suggested to occur to prevent depletion of the energy reserves (NAD and ATP) that are thought to be required for the later stages of apoptosis. Cleavage of PARP-1 has also been suggested to prevent futile repair of DNA strand breaks during the death program. We have shown previously that expression of a caspase-3-resistant PARP-1 mutant (mut-PARP-1) in osteosarcoma cells or PARP-1^{-/-} fibroblasts, increases the rate of cell death as a result of excessive NAD depletion (16). Herceg and Wang (17) also showed that expression of a similar PARP-1 mutant switches the mode of cell death induced by TNF from apoptosis to necrosis. To avoid excessive depletion of energy reserves and a switch to necrosis, cells exposed to inducers of apoptosis thus cleave PARP-1 into inactive peptides (17, 32). Although nonmodified PARP-1 is cleaved by caspase-3 (19, 33), automodified PARP-1 is preferentially cleaved by caspase-7 (34), thus emphasizing the necessity for PARP-1 cleavage during apoptosis. We now provide evidence for an additional role of PARP-1 and its cleavage in apoptosis. We have thus shown that, in response to the apoptosis inducer etoposide, PARP-1 mediates the covalent modification and consequent inhibition of the Ca²⁺, Mg²⁺-endonuclease DNAS1L3 and that subsequent internucleosomal DNA fragmentation mediated by this endonuclease occurs only after PARP-1 cleavage and hydrolysis of the PAR attached to DNAS1L3.

We took advantage of the previously described caspase-3-resistant PARP-1 mutant (mut-PARP-1) (16), the availability of DNAS1L3 cDNA (12), and human osteosarcoma cells that were shown not to express DNAS1L3 to examine directly the role of PARP-1 cleavage and consequent inactivation by caspases in the activation of DNAS1L3 both *in vitro* and *in vivo*. We thus demonstrated that cleavage of PARP-1 by caspase-3 *in vitro* prevented PARP-1-mediated inhibition of DNAS1L3 endonuclease activity. The failure of caspase-3 to prevent inhibition of DNAS1L3 endonuclease activity by the caspase-3-resistant mut-PARP-1 showed that PARP-1 cleavage by caspases is required for DNAS1L3-mediated DNA fragmentation. Conversely, these results indicate that the catalytic activity and structural integrity of PARP-1 are required for inhibition of DNAS1L3 activity by maintaining the endonuclease in a stable poly(ADP-ribosyl)ated state.

Human osteosarcoma cells provided a model system for our investigation into the physiological role of PARP-1 and its cleavage in DNAS1L3-mediated internucleosomal DNA fragmentation during apoptosis. These cells were thus shown not to express DNAS1L3, as assessed by RT-PCR analysis, and they failed to undergo internucleosomal DNA fragmentation in response to etoposide. No DNA fragmentation was observed in these cells even after extended incubation (36–48 h) with etoposide (data not shown). These cells do, however, undergo internucleosomal DNA fragmentation in response to staurosporine (16) or during confluency-triggered spontaneous apoptosis (19). We and others (19) used these cells undergoing such spontaneous apoptosis to identify and isolate caspase-3 (apopain) and to develop inhibitors of this protease. We subsequently described a transient burst of PAR synthesis that occurs in the cells early in apoptosis. With the use of PARP-

1^{-/-} immortalized fibroblasts, we showed that this early activation of PARP-1 is required for apoptosis induced by Fas and cycloheximide (35).

In the present study, osteosarcoma cells transfected with an expression vector encoding DNAS1L3 were shown to undergo internucleosomal DNA fragmentation in response to etoposide, suggesting that this drug specifically activated the endonuclease. This specific activation may be attributable to the requirement of DNAS1L3 for Ca²⁺ and the induction by etoposide of intracellular Ca²⁺ release (4, 36, 37). This hypothesis was supported by our observation that etoposide-induced DNA fragmentation in osteosarcoma cells expressing recombinant DNAS1L3 was inhibited by BAPTA, a blocker of intracellular Ca²⁺ release. Similarly, BAPTA inhibited etoposide-induced internucleosomal DNA fragmentation in cell types, such as human U-937 monocytes, mouse primary thymocytes, and splenocytes that express DNAS1L3 at a relatively high level (data not shown).

To examine the role of PARP-1 cleavage in DNAS1L3-mediated internucleosomal DNA fragmentation, we transfected osteosarcoma cells with plasmids encoding both DNAS1L3 and the caspase-3-resistant mut-PARP-1. We had previously shown that mut-PARP-1 remains intact after the induction of apoptosis by either staurosporine or the combination of TNF and cycloheximide in osteosarcoma and PARP-1^{-/-} cells, respectively (16) (data not shown). The mutant protein also remained intact in transfected osteosarcoma cells treated with etoposide (Fig. 4C). Expression of mut-PARP-1 completely blocked etoposide-induced, DNAS1L3-mediated internucleosomal DNA fragmentation, demonstrating that cleavage of PARP-1 and its consequent inactivation are required for DNAS1L3 endonuclease activity in osteosarcoma cells. Inactivation of PARP-1, however, may not be sufficient for activation of DNAS1L3, which likely also requires the degradation of the attached PAR moieties by PAR glycohydrolase.

Although the molecular mechanism of inhibition of DNAS1L3 by PARP-1 is not well established, the attachment of long, branched chains of ADP-ribose (38, 39) to the endonuclease by PARP-1 likely results in a marked decrease in its binding affinity for DNA (14). Such a reduction in binding affinity probably results from repulsion between the negative charges associated with both PAR and DNA. We and others (14, 30, 40, 41) have shown previously that PARP-1 substrates, including RNA polymerase α , DNA ligase, and the transcription factor p53, lose their ability to bind DNA when modified by poly(ADP-ribosylation).

DFF45 cleavage by caspase-3 and the resulting activation of DFF40 endonuclease activity are thought to be crucial for apoptotic DNA fragmentation (5, 9–11). Our results, however, indicate that DFF40 expression and cleavage of DFF45 are not sufficient to cause internucleosomal DNA fragmentation in osteosarcoma cells during etoposide-induced apoptosis. Our observation is consistent with several reports (1, 42, 43) indicating the insufficiency of DFF45 cleavage for apoptotic internucleosomal DNA fragmentation in several cell lines. These results, however, do not exclude the requirement of the DFF system in DNA fragmentation. In fact, we and others (3, 44, 45) have shown recently that both DFF45 and DFF40 are required for the processing of DNA into ~50-kb fragments in response to a variety of inducers including etoposide. Processing of genomic DNA into ~50-kb fragments is thought to be required for subsequent DNA fragmentation (46, 47). Indeed, osteosarcoma cells displayed ~50-kb DNA fragments after etoposide treatment suggesting that they were a direct result of DFF40 endonuclease activity after DFF45 cleavage. We have found that a variety of cell lines that fail to exhibit internucleosomal DNA

fragmentation all degrade their DNA into ~50-kb DNA fragments concomitantly with cleavage of DFF45 (data not shown). This further suggests that DFF40 endonuclease activity is only responsible for ~50-kb DNA fragmentation in some cells. Furthermore, DNAS1L3 endonuclease activity was observed to require DFF40 and DFF45 expression (unpublished observations), which suggests a cooperative activity between DFF40 and DNAS1L3.²

Expression of DNAS1L3 accelerated the death process both in osteosarcoma cells in the present study (data not shown) as well as in fibroblasts (13). This effect of the endonuclease might be attributable to the increased generation of DNA strand breaks resulting in increased PARP-1 activation and a consequent faster depletion of cellular energy (NAD and ATP) (16, 48). We and others (2, 3, 44) have recently shown that DFF45^{-/-} cells, which lack the ability to generate both 50-kb and oligonucleosomal DNA fragments, exhibit increased resistance to the induction of apoptosis by a variety of stimuli. The absence of such DNA fragmentation protected the cells from excessive activation of PARP-1 and thereby prevented depletion of intracellular NAD (3). The delay apparent in PARP-1 activation correlated with delays both in caspase-3 activation and in pro-apoptotic mitochondrial events, including loss of the mitochondrial membrane potential and the release of cytochrome *c*. On the basis of these results, we proposed that the generation of DNA fragments, together with PARP-1, mitochondria, and caspase-3, contributes to an amplification phase of apoptosis (3).

In conclusion, our present results suggest that PARP-1 and its cleavage by caspases play an important role in internucleosomal DNA fragmentation through inhibition and subsequent release from inhibition of DNAS1L3. Our data further demonstrate that the activation of PARP-1 and its inactivation by caspases are not passive events in apoptotic cell death but rather contribute directly to apoptotic DNA fragmentation.

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