Caspase-3-mediated Processing of Poly(ADP-ribose) Glycohydrolase during Apoptosis*

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Poly(ADP-ribose) glycohydrolase (PARG) is responsible for the catabolism of poly(ADP-ribose) synthesized by poly(ADP-ribose) polymerase (PARP-1) and other PARP-1-like enzymes. In this work, we report that PARG is cleaved during etoposide-, staurosporine-, and Fas-induced apoptosis in human cells. This cleavage is concomitant with PARP-1 processing and generates two C-terminal fragments of 85 and 74 kDa. In vitro cleavage assays using apoptotic cell extracts showed that a protease of the caspase family is responsible for PARG processing. A complete inhibition of this cleavage was achieved at nanomolar concentrations of the caspase inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde, suggesting the involvement of caspase-3-like proteases. Consistently, recombinant caspase-3 efficiently cleaved PARG in vitro, suggesting the involvement of this protease in PARG processing in vivo. Furthermore, caspase-3-deficient MCF-7 cells did not show any PARG cleavage in response to staurosporine treatment. The cleavage sites identified by site-directed mutagenesis are DEID256 ↓ V and the unconventional site MDVD297 ↓ N. Kinetic studies have shown similar maximal velocity (V max) and affinity (K m) for both full-length PARG and its apoptotic fragments, suggesting that caspase-3 may affect PARG function without altering its enzymatic activity. The early cleavage of both PARP-1 and PARG by caspases during apoptosis suggests an important function for poly(ADP-ribose) polymerase in the maintenance of the DNA integrity (1, 2). Recently, other pADPr synthesizing enzymes were identified, suggesting the presence within mammalian cells of a PARP-1-like enzyme family. A protein named tankyrase with homology to ankyrins and to the catalytic domain of PARP-1 was isolated from human tissue and shown to be associated with telomeres (3). Other proteins homologous to the catalytic domain of PARP-1 have also been reported (4–8).

Cells display a low basal level of pADPr, which can increase dramatically in response to DNA damaging agents (9–11). This increase in pADPr synthesis is transient and is followed by a rapid degradation by poly(ADP-ribose) glycohydrolase (PARG) (10, 12, 13). Two forms of PARG (74 and 59 kDa) have previously been purified from various tissues (14–19). However, the PARG cDNA recently isolated encodes an active protein of 111 kDa (20). Furthermore, we have recently reported the presence of only the 111-kDa form of PARG, which is localized mostly in the cytoplasm of the cells (21, 22). These findings raise questions about the cellular mechanism of PARG catabolism and the physiological significance of the 59- and 74-kDa forms of PARG.

Programmed cell death, or apoptosis, is an essential mechanism for appropriate embryogenesis, normal cell turnover, and the selection of lymphocytes (23, 24). Apoptosis is characterized by the activation of a cascade of cysteine proteases, named caspases, which trigger the biochemical and morphological changes occurring during cell dismantling. This includes nuclear condensation, DNA fragmentation, and the formation of apoptotic bodies (25, 26). Caspases cleave a specific set of cellular proteins like the lamins (27) and the inhibitor of caspase-activated deoxyribonuclease (28) that results in their inactivation. Caspase cleavage can also result in a constitutive activation of proteins such as protein kinase C δ (29, 30) as well as their own precursors, procaspases (31).

Cells undergoing apoptosis show a transient synthesis of pADPr (32, 33). We have previously reported that PARP-1 is cleaved by caspases during apoptosis (34–36). This cleavage separates its DNA-binding domain from the catalytic domain, resulting in the inactivation of the enzyme. Furthermore, we have recently shown that during etoposide (VP-16)-induced apoptosis, a transient pADPr synthesis is concomitant with the cleavage and inactivation of PARP-1 (37). This suggests a regulation of pADPr metabolism during apoptosis, which could also involve a modulation of PARG activity. In fact, PARG contains potential caspase cleavage sites, raising the possibility that this enzyme could also be cleaved during apoptosis. In this report we show that PARG is cleaved by caspase-3 during apoptosis. This cleavage releases enzymatically active C-terminal fragments of 85 and 74 kDa in human cells.

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‡ The abbreviations used are: PARP, poly(ADP-ribose) polymerase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; DEVD-pNA, acetyl-Asp-Glu-Val-Asp-p-nitroanilide; etoposide, VP-16; GFF, green fluorescent protein; MNNNG, 1-methyl-5-nitro-1-nitroguanidine; PAGE, polyacrylamide gel electrophoresis; pADPr, poly(ADP-ribose); PARG, poly(ADP-ribose) glycohydrolase; PMSF, phenylmethanesulfonylfluoride; TVAD-CHO, acetyl-Tyr-Val-Ala-Asp-aldehyde; 5-DEVD-fmk, 5-acetyl-Asp-Glu-Val-Asp-fluoromethyl ketone.

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EXPERIMENTAL PROCEDURES

Materials—[35]P[NaD (30 Ci/mmol) and [35]methionine (1175 Ci/mmol) were purchased from PerkinElmer Life Sciences. VP-16, staurosporine, activated calf thymus DNA, 1,5-isooquinolinodiol, and 1-methyl-3-nitro-1-nitroguanidine (MNNG) were obtained from Sigma-Aldrich. β-NAD and the protease inhibitor mixture tablets were from Roche Molecular Biochemicals. The tetrapeptide caspase inhibitors, acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) and acetyl-Tyr-Ala-Asp-aldehyde (YVAD-CHO), and the colorimetric substrate acetyl-Asp-Glu-Val-Asp-nitroanilide (DEVD-pNA) were purchased from Biomol Research Laboratories. z-Acetyl-Asp-Glu-Val-Asp-fluoromethyl ketone (z-DEVD-fmk) was from Calbiochem-Novabiochem. Anti-fas antibody was supplied by PanVera Inc. Calf thymus PARP-1 was purified as described previously (38). Recombinant human caspase-3, -6, and -7 were prepared as described previously (39).

Cell Culture and Treatments—Human leukemia HL-60 and Jurkat cells, breast carcinoma MCF-7, bovine kidney MDBK, mouse embryo C3H10T1/2, and monkey kidney Cos-7 cells were grown according to American Type Culture Collection instructions. The cells were treated with the apoptotic inducers in a complete growth medium at the indicated concentrations for different lengths of time. The cell permeable irreversible caspase inhibitor z-DEVD-fmk (3 μM) was added to the culture medium 30 min before treatment.

Western Blots and Preparation of Apoptotic Cell Extracts—After drug treatment, cells were washed with ice-cold HEPES-saline buffer A (10 mM HEPES, pH 7.4, 140 mM NaCl, 7 mM KCl, 6 mM glucose) and lysed in the ice-cold hypotonic buffer B (25 mM HEPES, pH 7.5, 1 mM EGTA, 5 mM MgCl2, 0.1% Triton X-100, 250 μM pMSF, 2 mM diithiothreitol, anti-fas antibody (1:1000 dilution), and aliquots were taken for protein determination (40). The remaining cell extract was kept with reducing loading buffer or homogenized in buffer B by 20 strokes in a Dounce homogenizer and centrifuged at 15,000 × g for 60 min at 4 °C to obtain the apoptotic cell extract. DEVDase activity was routinely assayed in the apoptotic cell extract using the substrate DEVD-pNA to obtain the apoptotic cell extract. DEVDase activity was assayed as described previously (38). Recombinant human caspase-3, -6, and -7 were used as specific markers of endoplasmic reticulum, was detected with an anti-calnexin monoclonal antibody (Molecular Probes). Cytochrome oxidase marker of mitochondria, was detected with a monoclonal anti-cytochrome oxidase monoclonal antibody (Molecular Probes). Lac-tate dehydrogenase and 5-nucleotidase were used as specific markers for cytoplasm and plasma membrane, respectively. Both enzyme activities were determined with kits from Sigma.

Protease Cleavage Assay—A bovine PARG cDNA lacking the region coding for the N-terminal 74 amino acids (21) was used to translate the PARG in vitro with the transcription/translation TNT kit (Promega) in the presence of [35]methionine. The translation mixture (5 μl) was incubated in a total volume of 12.5 μl for 3 h at 37 °C, either with purified caspases resuspended in buffer C (50 mM HEPES, pH 7.4, 100 mM NaCl, 10% sucrose, 10 mM EDTA, and 1.6 mM CHAPS) or with the apoptotic cell extract diluted in buffer B. The reaction was stopped by addition of reducing loading buffer, and the digestion products were analyzed by SDS-PAGE and autoradiography. The amount of radioactivity was determined using PhosphorImager and the Instant Image Analyzer (Packard Instrument Company). For the cleavage inhibition assays, the tetrapeptide inhibitor DEVD-CHO or YVAD-CHO was added to the apoptotic cell extract at the indicated concentrations.

Mapping of the Caspase Cleavage Sites in PARG by Mutagenesis—Site-directed mutagenesis was achieved using the QuikChange kit (Stratagene). The human DEID sequence in positions 253–256 was introduced into the corresponding region (positions 254–257) of the bovine protein (replacing the bovine EEDV site) using the primer 5′-CAGGGTGTCACGAGCAAGGAGATACCTGTGGTTCCG-3′. The ascitic acid in position 308 of bovine PARG was mutated to alanine using the primer 5′-GAGTACCAATGTTGATCTAATTTTACAAAAAATGTGTTACGGG-3′. The mutations were confirmed by DNA sequencing.

Analysis of pADPr Levels—pADPr determination was done using the immunodot blot technique as described previously (45). Briefly, dihydroxyboryl Bio-Rex purified pADPr was diluted in 0.4 mM NaOH containing 10 mM EDTA and loaded on Hybond N+ membrane using a dot-blot manifold system (Life Technologies, Inc.). The presence of pADPr was detected using a peroxidase-conjugated anti-rabbit IgG and chemiluminescence reaction. Quantification was performed using a cooled CCD camera equipped with a Chemi Imager 4000, and the data were analyzed with the Digital Imaging and Analysis Systems (Alpha Innotech Inc.).

Partial Purification of PARG and Caspase-3 Cleavage Assay—Jurkat cells (5 × 10⁶) were sedimented at 1,900 × g for 5 min at 4 °C and washed with buffer A. The cell pellet was resuspended in 10 ml of ice-cold buffer (20 mM KPO4, pH 7.4, 1 mM EGTA, 5 mM MgCl2, 5 mM β-mercaptoethanol, 0.5 mM pMSF, antiprotease mixture) and homogenized with 20 strokes using a Dounce homogenizer. Cell homogenates were centrifuged at 100,000 × g for 60 min at 4 °C. The supernatant was completed with 10 ml of buffer (20 mM KPO4, pH 7.4, 800 mM KCl, 1 mM EDTA, 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2% glycerol, 5% β-mercaptoethanol, 0.5 mM pMSF, antiprotease mixture) and mixed with 5 ml of Redagarose preswollen resin (Amicon Corp.) equilibrated with buffer D (20 mM KPO4, pH 7.4, 400 mM KCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 0.5 mM pMSF, antiprotease mixture). After 1 h of gentle shaking at 4 °C, the mixture was washed with buffer D and with the same buffer containing 600 mM of KCl, and the enzyme was eluted with buffer D containing 1–2 mM KCl. The active fraction was desalted with four columns of Sephadex G-25 resin (Amersham Pharmacia Biotech) packed in a 60-ml syringe (5 × 7 cm) using buffer E (50 mM Tris-HCl, pH 9.0, 150 mM KCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 0.5 mM pMSF, antiprotease mixture) and applied on 5 ml of prepacked Heparin Sephrose (HiTrap Heparin, Amersham Pharmacia Biotech) equilibrated with the same buffer. The eluent was done with buffer E containing 200–300 mM KCl. The active fraction was adjusted to pH 7.4 and applied on a 1 × 3-cm phosphocellulose column (Whatman) equilibrated with buffer F (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 0.5 mM pMSF, antiprotease mixture). After washing with buffer F containing 100–300 mM KCl, the elution was collected in the same buffer containing 200–300 mM KCl. The active fraction was used for DHB-Sepharose-polymer affinity chromatography as described previously (17) except that the pADPrs used for the purification was synthesized without the formic acid resuspension and trichloroacetic acid precipitation step, and the elution was done at pH 7.4. Partially purified PARG (550 ng) was cleaved by caspase-3 (200 or 600 ng) in a total volume of 600 μl of buffer C without NaCl. After 3 h of incubation, the reaction was stopped by the addition of SDS at a concentration of 0.9 N acetic acid. The TLC plate was electronically autoradiographed on the Instant Image Analyzer as described above. The radioactive in the ADP-ribosyl spot, and the origin was used to calculate the activity. The amount of pADPr was quantified using the Radioisotope Analyzing System (Packard Instrument Company). The amount of pADPr was determined using various concentrations of [32P]pADPr (0.1, 0.3, 1, 3, 10, and 30 μM) to establish Lineeweaver-Burk reciprocal plots.

One-dimensional PARG Activity Zymogram—An activity zymogram consisting of an SDS-PAGE of PARG in a gel containing [32P]automodified PARP-1 was done according to Brochu et al. (46). After renaturation, the presence of PARG was determined by the disappearance of

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treatments, cell lysates (40 μg of protein) were resolved using 8% SDS-PAGE and subsequently analyzed by immunoblotting using the polyclonal anti-PARG C-terminal G-03 antibody and the monoclonal anti-PARP-1 CII10 antibody. Jurkat cells were treated with 120 μM VP-16 (A) or 300 nM staurosporine (B). The irreversible caspases inhibitor z-DEVD-fmk (3 μM) was added to the culture medium 30 min before treatment. C, HL-60 cells treated with 88 μM VP-16. D, Jurkat cells treated with 1 μg/ml of anti-Fas antibody. The results are representative of three independent treatments. DMSO, dimethyl sulfoxide; MW, molecular mass.

FIG. 1. Cleavage of PARG during apoptosis. Jurkat and HL-60 cells were treated with apoptotic inducers for the indicated times. Following treatments, cell lysates (40 μg of protein) were resolved using 8% SDS-PAGE and subsequently analyzed by immunoblotting using the polyclonal anti-PARG C-terminal G-03 antibody and the monoclonal anti-PARP-1 CII10 antibody. Jurkat cells were treated with 120 μM VP-16 (A) or 300 nM staurosporine (B). The irreversible caspases inhibitor z-DEVD-fmk (3 μM) was added to the culture medium 30 min before treatment. C, HL-60 cells treated with 88 μM VP-16. D, Jurkat cells treated with 1 μg/ml of anti-Fas antibody. The results are representative of three independent treatments. DMSO, dimethyl sulfoxide; MW, molecular mass.

RESULTS

Cleavage of Endogenous PARG during Apoptosis—Human Jurkat leukemia cells were treated with the topoisomerase II inhibitor VP-16 or the PKC inhibitor staurosporine and analyzed by Western blot for PARG cleavage (Fig. 1, A and B). In untreated cells, PARG was present in two bands of ∼111 kDa corresponding possibly to post-translational modifications or different translation origins. In cells undergoing apoptosis, these bands were cleaved into ∼85- and 74-kDa fragments at approximately the same time as PARP-1 cleavage. The two apoptotic fragments are in the C-terminal region of the protein because they are recognized by the anti-peptide antibody.

PARG cleavage could be inhibited by the cell permeable caspases inhibitor z-DEVD-fmk, suggesting the involvement of caspases in PARG cleavage. PARG was also cleaved into the ∼85- and 74-kDa fragments in HL-60 cells treated with VP-16 (Fig. 1C) and Jurkat cells treated with anti-Fas antibody (Fig. 1D) or the ligand Trail (data not shown). These results suggest that PARG processing is a general feature of apoptosis.

Because pADPr synthesis occurs in apoptotic cells (32, 33, 37), we asked whether PARG cleavage is specific to apoptosis or whether it could also occur during the early pADPr metabolism in response to DNA strand breaks. HL-60 cells were treated with MNNG, and the pADPr level was determined by the immunodot-blot method (Fig. 2A). MNNG treatment induced a transient synthesis of pADPr, which was abolished by the PARP inhibitor 1,5-isoquinolinediol. Western blot analysis of PARG showed that the protein remained unaffected during MNNG treatment (Fig. 2B), unlike following VP-16 treatment. This indicates that, in living cells, pADPr catabolism does not require PARG cleavage.

Cleavage of PARG by Caspase-3 during Apoptosis—To determine the nature of the protease responsible for PARG cleavage during apoptosis, in vitro translated bovine PARG was incubated with different amounts of apoptotic HL-60 cell extract (Fig. 3A). Two PARG translation products, corresponding to 103 and 91 kDa were observed in the control. These bands probably correspond to two translation start points from the partial PARG cDNA. Following the addition of the apoptotic cell extract, PARG was cleaved to a 74-kDa fragment in a dose-dependent manner, suggesting that an apoptotic protease is responsible for this cleavage. The 85-kDa fragment produced in human cells (Fig. 1) was not observed with bovine PARG.
Absence of PARG cleavage during pADPr metabolism. A, HL-60 cells were treated with 50 μM MNNG and pADPβS was detected by immunodot-blot using LP96–10 antibody (closed circles). As control, cells were pretreated with 1 mM 1,5-isoquinolinediol for 5 min before MNNG treatment (open circles). B, HL-60 cells were treated with 50 μM MNNG or 68 μM VP-16, and cell lysates (40 μg of protein) were immunoblotted for PARG using G-03 antibody. The results are representative of four independent experiments. DMSO, dimethyl sulfoxide; MW, molecular mass.

Similar results were obtained with cytosolic extracts from Jurkat cells treated with staurosporine or anti-Fas antibody (data not shown). The small N-terminal fragments derived from the cleavage of the 103- and 91-kDa products were hardly detectable because of their low radioactivity (data not shown). To study the involvement of caspases in PARG cleavage, the specific tetrapeptide caspase inhibitors DEVD-CHO and YVAD-CHO were used (Fig. 3B). The cleavage of PARG was completely inhibited by 50 nM of DEVD-CHO but only partially inhibited by 500 nM of YVAD-CHO, suggesting that a caspase-3-like activity present in apoptotic HL-60 cells is involved in PARG processing. Similar patterns of inhibition were obtained with cytosolic extracts from Jurkat cells treated with staurosporine or anti-Fas antibody (data not shown). To further determine which caspase is responsible for PARG cleavage, in vitro translated bovine PARG was incubated with caspase-3, -6, and -7. Using 4 ng of purified caspases per assay (−7 nM), a weak cleavage of PARG was observed with caspase-3. Caspase-6 and -7 did not produce detectable cleavage at this concentration. At 20 ng/assay (−35 nM), PARG was cleaved to a significant degree by caspase-3 and marginally by caspase-7 (Fig. 3C). These results suggest that caspase-3 could be responsible for PARG cleavage in vitro. To test this hypothesis, caspase-3-deficient MCF-7 cells were used to determine whether PARG could be cleaved in the absence of this protease following apoptotic treatment (Fig. 3D). In this cell line, PARG was not cleaved in response to staurosporine treatment, whereas PARP-1 was almost totally cleaved.

Identification of PARG Cleavage Sites—In vitro translated bovine PARG was cleaved only into a 74-kDa fragment, whereas human PARG was cleaved into 85- and 74-kDa fragments (Figs. 1 and 3). Analysis of the PARG sequence (Fig. 4A) revealed the presence of a potential DEID motif, present only in human PARG, that could generate the 85-kDa fragment. To determine whether the 85-kDa fragment is produced in other mammalian cell types, cell extracts from MDBK (bovine), C3H10T1/2 (mouse), and Jurkat (human) cells were treated with caspase-3 and assayed for PARG cleavage with anti-PARG antibody (Fig. 4B). PARG was cleaved to its 74-kDa fragment in all cell types, but only human and mouse PARG showed the presence of additional fragments (of 85 and 66 kDa, respectively). In MDBK cells, three faint bands migrating below 66 kDa are present in the control as well as the treated extract and are thus unrelated to caspase cleavage. To determine whether the human DEID sequence corresponds to the caspase cleavage site that generates the 85-kDa fragment in human cells, we introduced this sequence in the corresponding region of bovine PARG. The resulting PARG mutant construct was translated in vitro and subjected to a cleavage assay using recombinant caspase-3 or the apoptotic cell extract (Fig. 4C). Cleavage of the mutant PARG produced the 85-kDa fragment in addition to the 74-kDa fragment. This cleavage was also obtained with the apoptotic cell extract and was completely inhibited by 50 nM of DEVD-CHO but not by 500 nM of YVAD-CHO. To determine the caspase-3 cleavage site that produces the 74-kDa fragment, several aspartic acid residues were mutated to alanine. Mutation at amino acid position 308 of bovine PARG abolished the cleavage of PARG by caspase-3 (Fig. 4D). This cleavage site, MDVD, is conserved in mouse, bovine, and human PARG (Fig. 4A). Mutations of other aspartic acid resi-
cytosolic extracts (40 μg of protein) prepared from MBDK (bovine), C3H10T1/2 (mouse), and Jurkat (human) cells were treated with 40 ng of caspase-3 for 3 h at 37 °C and immunoblotted for PARG with G-03 antibody. The sequence DEID, present only in human PARG, was introduced into the corresponding region of bovine PARG. The resulting PARG mutant construct was translated in vitro and subjected to cleavage by recombinant caspase-3 (20 ng) or apoptotic cytosolic extract (20 μg of protein) prepared from HL-60 cells treated with 68 μM VP-16. The inhibitors DEVD-CHO and YVAD-CHO were used at 50 and 500 nM, respectively.

To determine whether PARG cleavage results in a change in its catalytic function, enzymatic activity was measured in total extracts of HL-60 cells at various times after VP-16 treatment. PARG activity was not altered for up to 360 min of treatment (Fig. 5A). A PARG activity gel was therefore used to determine whether the proteolytic products of PARG were active. The 85- and 74-kDa fragments still possess the capacity to hydrolyze the pADPr like the full-length enzyme (Fig. 5B). Similar results were obtained in Jurkat cells treated with staurosporine or anti-Fas antibody (Fig. 5C). To further characterize the kinetic parameters of PARG and its apoptotic fragments, we purified 848-fold full-length PARG from a cytosolic fraction of Jurkat cells (Fig. 6A). The purified enzyme was used for in vitro cleavage by caspase-3 followed by PARG activity assay. A representative blot shown in Fig. 6B indicates that almost all the full-length PARG is cleaved to generate the 85- and 74-kDa fragments. Kinetic analysis showed similar $K_m$ and $V_{max}$ for PARG and a mixture of the apoptotic fragments, 85 and 74 kDa (Fig. 6C). The complete cleavage of PARG to the 74-kDa fragment did not result in a significant change of the $K_m$ and $V_{max}$ (Fig. 6B and C). The DEVD-CHO added to stop the caspase-3 reaction did not interfere with the PARG assay, because its addition to the reaction mixture did not inhibit the enzyme activity (data not shown). These results corroborate those obtained with PARG activity determination in total cell extract and by activity gel.

**Cellular Localization of PARG and of Its 74-kDa Apoptotic Fragment**—Because the cleavage of PARG does not alter its catalytic activity, we examined whether it could result in a change of its cellular localization. Homogenates from control and apoptotic Jurkat cells fractionated into S-100 cytosol and pellet containing nuclei and the other organelles were analyzed for the presence of PARG by immunoblotting (Fig. 7A). In control and apoptotic Jurkat cells, PARG was mostly associated to the cytosolic fraction, which contains most of the lactate dehydrogenase activity. The organelles fraction containing nu-

**Fig. 4. Mapping of caspase cleavage sites on PARG.** A, schematic representation of PARG structure and the putative cleavage sites. B, cytosolic extracts (40 μg of protein) prepared from MBDK (bovine), C3H10T1/2 (mouse), and Jurkat (human) cells were treated with 40 ng of caspase-3 for 3 h at 37 °C and immunoblotted for PARG with G-03 antibody. C, the sequence DEID, present only in human PARG, was introduced into the corresponding region of bovine PARG. The resulting PARG mutant construct was translated in vitro and subjected to cleavage by recombinant caspase-3 (20 ng) or apoptotic cytosolic extract (20 μg of protein) prepared from HL-60 cells treated with 68 μM VP-16. The inhibitors DEVD-CHO and YVAD-CHO were used at 50 and 500 nM, respectively. D, the aspartic acid residue at amino acid position 308 of bovine PARG was mutated to alanine. The resulting PARG mutant construct was translated in vitro and subjected to cleavage by 20 ng of caspase-3. The results are representative of three independent experiments. MW, molecular mass; NLS, nuclear localization signal.

**Fig. 5. PARG activity during apoptosis.** A, PARG activity in a total cell extract from HL-60 cells treated with 68 μM VP-16 (closed triangle) or dimethyl sulfoxide (open circle). Data are the means ± S.D. of three experiments. B, activity zymogram consisting of SDS-PAGE followed by the renaturation of proteins using a gel containing PARP-1-bound [$^{32}$P]pADPr. Total cell extracts (20 μg of protein) from HL-60 cells treated with 68 μM VP-16 were prepared at the indicated time as for Western blot. C, activity zymogram of the total cell extract (20 μg of protein) from Jurkat cells treated with 1 μM staurosporine or 1 μg/ml of anti-Fas antibody for 3 h. The results are representative of three independent experiments. DMSO, dimethyl sulfoxide; MW, molecular mass. nuclei, mitochondria, endoplasmic reticulum and plasma membranes did not contain a significant amount of PARG or its apoptotic fragments. To further directly monitor the cellular localization of PARG during apoptosis, GFP was fused to the N terminus of both PARG, and the 74-kDa apoptotic fragment and the resulting recombinant proteins were transiently expressed in Cos-7 cells (Fig. 7B). In the absence of treatment, both GFP-PARG and GFP-74 showed predominant cytoplasmic localization (Fig. 7C), whereas GFP was localized in the nucleus and in the cytoplasm as expected. The localization of GFP-PARG is consistent with the previously reported distribution for PARG (22). Cos-7 cells treated with staurosporine for 7 h underwent apoptosis as determined by the typical apoptotic...
morphological changes and about 50% of PARP-1 cleavage (data not shown). Only the cells with an apoptotic morphology were analyzed for PARG distribution by fluorescence microscopy. Following staurosporine treatment, most of the fluorescence remained in the cytoplasm, and no detectable redistribution of PARG or its 74-kDa fragment to the nucleus was observed (Fig. 7C). These results suggest that the active 74-kDa fragment of PARG may play a role in the cytoplasm of apoptotic cells.

**DISCUSSION**

Two forms of PARG, 59 and 74 kDa, have been previously purified from mammalian cells. However, bacterial expression of the bovine PARG cDNA recently isolated, produced a protein of 111 kDa (20). We recently showed that under normal growth conditions, PARG is present only as a doublet at 111 kDa with a cytoplasmic localization (21). These findings raised questions about the biological significance and the roles of the 59- and the 74-kDa forms of PARG. In this study we show that, during apoptosis, PARG is specifically cleaved by caspase-3 to produce a catalytically active 74-kDa fragment in different mammalian species.

We established here a new purification procedure that allowed us to isolate for the first time the full-length PARG from mammalian cells (Fig. 6). Purified PARG under these conditions did not contain its 59-kDa form previously reported (14, 17, 20). This C-terminal 59-kDa fragment of PARG was not observed during apoptosis or normal pADPr metabolism (Figs. 1 and 2). By using previously reported methods (17) to purify PARG from Jurkat cells, we noticed that the 59-kDa fragment was produced despite the use of an antiprotease mixture. During later steps of purification, the 111-kDa PARG was totally cleaved to 59 kDa (data not shown), indicating that the enzyme contains a site that is very sensitive to proteases. However, whether this fragment of PARG could be produced in vivo in particular circumstances remains to be determined.

Human PARG is cleaved during Fas receptor-, staurosporine- and VP-16-induced apoptosis to generate fragments of 85 and 74 kDa, suggesting that this cleavage is a common feature of apoptosis. The involvement of caspase-3 in PARG cleavage was evidenced using an *in vitro* cleavage assay in the presence of apoptotic cell extract, specific caspases inhibitors, and recombinant caspases. PARG cleavage by caspase-7 was negligible, despite the fact that caspase-3 and caspase-7 are execution caspases with similar specificity and $K_m$ for the tetrapeptide DEVD-pNA (47). PARG was not cleaved in caspase-3-deficient MCF-7 cells during staurosporine-induced apoptosis, during which caspase-7 is known to be activated (37).

PARG was cleaved by caspase-3 to produce the 74-kDa fragment at the unconventional site MDVD that lacks an aspartic acid at the P4 position. However, previous inhibition studies using synthetic peptides have shown that this P4 aspartic acid is required for the cleavage by caspase-3 (48, 49). In contrast, our results support a recent report showing a noncanonical EEED site for caspase-3 in topoisomerase I (50). These results suggest that other parameters such as the tertiary structure and post-translational modification of proteins could also be involved in the specificity of cleavage by caspases. For instance, the sequence DEAD in the retinoblastoma protein is cleaved by caspase-7 but not by caspase-3 (51). In addition, we have recently observed that poly(ADP-ribosyl)ation of PARP-1 stimulates its cleavage by caspase-7 but not by caspase-3 (37). The DEID site that produces the 85-kDa fragment in human cells is quite similar to the typical sequence $^{10}$DXXD$^{13}$ for caspase-3 found in other proteins cleaved by this protease (48). However, this fragment is produced only in human cells, indicating that its production is not essential in other mammalian species. Further cleavage of this fragment by caspase-3 to a 74-kDa fragment could occur during apoptosis. On the other hand, we
cannot exclude the possibility that this fragment could have a specific role to play during apoptosis in human cells.

During apoptosis, many different enzymes are cleaved by caspases to dissociate their regulatory domain from their catalytic domain. This results in the inactivation or the constitutive activation of these proteins (31). Our results indicate that the C-terminal domain of PARG produced during apoptosis contains the active site of the enzyme and is able to hydrolyze pADPr in vitro (Figs. 5 and 6). This is consistent with previous results indicating that the PARG C-terminal 59-kDa fragment possesses glycohydrolase activity (20). The N-terminal domain is likely to be responsible for the regulation of PARG function in vivo. Because the glycohydrolase assay in our study was done with free pADPr, we could not rule out the possibility for distinct manners of action of PARG and its apoptotic fragments on poly(ADP-ribosyl)ated proteins in vivo. Indeed, some specific interactions between the N-terminal domain of full-length PARG and other proteins may occur that obviously are impossible for the apoptotic fragments. If the cleavage of PARG in mammalian apoptotic cells is to circumvent certain regulation of the enzyme function by the N-terminal domain, we could expect that the 74-kDa fragment would continuously catabolize pADPr and possibly obliterate its cellular functions (e.g. signaling of cellular stress). Consistent with this hypothesis,

![Fig. 7. Cellular localization of PARG during apoptosis.](image-url)
PARP-1 is cleaved during apoptosis resulting in the suppression of its pADPr synthesis function. The fact that the 74-kDa fragment remains mostly in the cytoplasm may suggest a role in relation with the other PARP-1-like proteins. In fact, recent studies have reported the presence of a new PARP (vPARP) associated with the ribonucleoproteins, vault particles in the cytoplasm (7). A cytoplasmic distribution was also reported for the telomerases associated PARP (tankyrase) (52, 53). On the other hand, we have previously shown that the nuclear accumulation of pADPr following MNNG treatment is abolished in PARG-overexpressing Cos-7 cells in comparison with the control vector transfected cells (22). However, overexpression of PARG results in its cytoplasmic localization without any detectable redistribution of the enzyme following MNNG treatment (22). This suggests that a very small but sufficient amount of PARG (determined indirectly by the rapid polymer degradation) is translocated to the nucleus and catalyzes pADPr. The same model could be applied to the 74 kDa fragment because it still possesses the putative nuclear localization signal (20). Further studies are necessary to understand the role of PARG cleavage during apoptosis. The generation of PARG-deficient mice and cells will be a useful model to study this role (e.g. reintroduction of an uncleavable PARG mutant into the enzyme-deficient cells).

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