Cleavage of Autamodified Poly(ADP-ribose) Polymerase during Apoptosis

EVIDENCE FOR INVOLVEMENT OF CASPASE-7*

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The abundant nuclear enzyme poly(ADP-ribose) polymerase (PARP) synthesizes poly(ADP-ribose) in response to DNA strand breaks. During almost all forms of apoptosis, PARP is cleaved by caspases, suggesting the crucial role of its inactivation. A few studies have also reported a stimulation of PARP during apoptosis. However, the role of PARP stimulation and cleavage during this cell death process remains poorly understood. Here, we measured the stimulation of endogenous poly(ADP-ribose) synthesis during VP-16-induced apoptosis in HL60 cells and found that PARP was cleaved by caspases at the time of its poly(ADP-ribose)ylation. In vitro experiments showed that PARP cleavage by caspase-7, but not by caspase-3, was stimulated by its automodification by long and branched poly(ADP-ribose). Consistently, caspase-7 exhibited an affinity for poly(ADP-ribose), whereas caspase-3 did not. In addition, caspase-7 was activated and accumulated in the nucleus of HL60 cells in response to the VP-16 treatment. Furthermore, caspase-7 activation was concomitant with PARP cleavage in the caspase-3-deficient cell line MCF-7 in response to staurosporine treatment. These results strongly suggest that, in vivo, it is caspase-7 that is responsible for PARP cleavage and that poly(ADP-ribose)ylation of PARP accelerates its proteolysis. Cleavage of the active form of caspase substrates could be a general feature of the apoptotic process, ensuring the rapid inactivation of stress signaling proteins.

Apoptosis is a conserved mechanism of cell death controlling the development and homeostasis of multicellular organisms. In the last few years, a family of cysteine proteases named caspases, highly related to the interleukin-1-converting enzyme and the proapoptotic CED-3 gene of Caenorhabditis elegans, have emerged as important mediators of the apoptotic process (1, 2). The 89-kDa fragment, carrying the automodification domain and the catalytic domain of the enzyme, retains only a weak ability to bind to inactive DNA damage. The 24-kDa apoptotic fragment of PARP irreversibly binds to DNA breaks, and its appearance is mediated by caspase-3 activation. The presence of this fragment is believed to be a general feature of the apoptotic process, ensuring the rapid inactivation of stress signaling proteins.

Three of these caspases have been implicated in the execution phase of apoptosis (1, 2) and are shown to cleave specific substrates as the cell begins to present the characteristic morphological changes of apoptosis (nuclear condensation, cell blebbing, and formation of the apoptotic bodies). Caspase-3 was shown to cleave a wide range of cytoplasmic and nuclear proteins (1), which suggests an important role for this protease in apoptosis. Strikingly, caspase-3 knock-out mice, while showing major defects of apoptosis in the brain, seemed to have normal apoptotic responses otherwise (3). Caspase-7 is highly related to caspase-3 and shows the same synthetic substrate specificity in vitro (4). It is believed that caspase-7 cleaves caspase-3 substrates in caspase-3 knock-out mice. A recent caspase-3 knockout report suggested that caspase-3 and -7 are distinct but possibly overlapping roles in apoptosis, because some caspase substrates are not cleaved in the knock-out cells, but the overall process is not altered (5). The third execution phase caspase, caspase-6, is responsible for the cleavage of the lamins (6).

Polyl(ADP-ribose) polymerase (PARP) synthesizes poly(ADP-ribose) from NAD in response to DNA strand breaks and is involved in many genomic processes including DNA base excision repair (7), DNA replication (8) and transcription (9). PARP is thought, along with DNA protein kinase, ATM, and p53, to be part of the cascade signaling DNA damage in the cell (10). PARP was one of the first substrates that was shown to be cleaved by caspases (11, 12). Although almost all caspases, including caspase-1, can cleave PARP in vitro (2, 13), it is most likely that caspase-3 and -7 are responsible for the in vivo processing of PARP to its apoptotic 24- and 89-kDa fragments (1, 2). The 89-kDa fragment, carrying the automodification domain and the catalytic domain of the enzyme, retains only a basal activity because it loses its capacity to bind to damaged DNA (11). The 24-kDa fragment, which contains the two zinc fingers responsible for the DNA binding of PARP, is very likely to act as a transdominant inhibitor of active PARP, similar to the inhibition observed with the complete 46-kDa DNA-binding domain (14). Indeed, Smulson et al. (15) have shown recently that the 24-kDa apoptotic fragment of PARP irreversibly binds to DNA breaks.

Here, we have studied the temporal association between PARP activation, its cleavage, and the appearance of the DNA ladder during the course of VP-16-induced apoptosis in HL60.

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The abbreviations used are: PARP, poly(ADP-ribose) polymerase; AFC, 7-amino-4-trifluoromethyl; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; CHO, aldehyde; DEVD, acetyl-aspartylglu-Val-asp; DHQ, 1,5-dihydroxyisoquinoline; pNA, p-nitroaniline; VP-16, etoposide.
caspase-7 but not by caspase-3. We also show that caspase-7 is activated in the nucleus in response to the VP-16 treatment in HL60 cells.

EXPERIMENTAL PROCEDURES

**Materials**—[^32P]NAD (111 Tbp/mmol) and a [32P]dCTP (111 Tbp/mmol) were purchased from NEN Life Science Products. DEVD-pNA, DEVD-AFC, and DEVD-CHO were purchased from Boekel Research Laboratory. DHQ was obtained from Aldrich. T4 DNA polymerase was purchased from Amersham Pharmacia Biotech. The polyclonal antibody against caspase-7 p19 subunit was kindly provided by Dr. G. M. Cohen (16). Peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch. Other reagents were obtained from Sigma or Roche Molecular Biochemicals.

**Cell Culture and Induction of Apoptosis**—HL60 human leukemia cells and MCF-7 cells were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine for MCF-7 cells. Apoptosis was induced with 68 mM VP-16 and 1 mM staurosporine for HL60 and MCF-7, respectively. For the Western blots, cells were centrifuged for 5 min at 800 × g and washed with HEPES buffer (140 mM NaCl, 7 mM KCl, 6 mM glucose, 10 mM HEPES, pH 7.4) and resuspended in reducing loading buffer. The cells were sonicated prior to loading on a 8% polyacrylamide gel, resolved, and transferred to a nitrocellulose membrane. PARP cleavage was detected using the monoclonal antibody C11-10, which recognizes full-length PARP and its 89-kDa apoptotic fragment, as described by Lazebnik et al. (17). The 116- and 89-kDa bands were quantified using a cooled CCD camera equipped with a Chemi Imager 4000, and the data were analyzed with the Digital Imaging Analysis Systems (Alpha Innotech Inc.). The apoptotic intercellular cleavage of the DNA was performed as described by McGahon et al. (18). For the separation of nucleus from the cytoplasm, cells were centrifuged in 10 mM Tris, pH 7.4, 1 mM EDTA, 300 mM sucrose, 2 mM β-mercaptoethanol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and antiprotease mixture (Roche Molecular Biochemicals). Cells were then homogenized with a Dounce homogenizer and centrifuged at 10,000 × g for 3 min. Pellets containing the nucleus were resuspended in the same buffer. Cytoplasm and nucleus were then diluted in reducing loading buffer prior to loading on a 15% polyacrylamide gel and submitted to Western blotting with an anti-caspase-7 p19 subunit antibody.

**Analysis of Cellular NAD and Poly(ADP-ribose) Levels**—To determine the NAD levels, cellular NAD was determined as described (25). One unit of caspase activity was defined as the amount of caspase necessary to produce one pmol of AFC/min from the substrate DEVD-AFC, in the same reaction conditions that for PARP.

**Noncovalent Interactions between Caspases and Poly(ADP-ribose): Polymer Blot Assay**—The experiments were carried out essentially as described by Althaus et al. (27). Caspases and total histones were resolved on a 15% acrylamide gel and transferred to a nitrocellulose membrane. The membrane was washed three times with TBS-T (10 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20), blocked with 3% bovine serum albumin in TBS-T, and washed again three times with TBS-T prior to incubation with the indicated quantities of [32P]poly(ADP-ribose) for 1 h. After washing the membranes with TBS-T, the radioactive polymer still bound to the membrane was analyzed by autoradiography. The same experiments were also carried out with radiolabeled genomic DNA (Sigma). The DNA was radiolabeled by [32P]dCTP using T4 DNA polymerase, as described by Legault et al. (28).

**RESULTS**

**PARP Cleavage by Caspases at the Time of Its Activation**—During apoptosis, a drop in cellular NAD was observed in some cellular systems. This NAD depletion was partially inhibited by the PARP inhibitor 3-aminobenzamide, thus implicating PARP activation in apoptosis (11, 29, 30). However, enzymes other than PARP have recently been shown to decrease NAD levels following DNA damage (31). Furthermore, studies on polymer levels during apoptosis have relied on permeabilized cells, which do not allow an accurate measurement of endogenous poly(ADP-ribose) levels. These techniques have thus failed to evaluate the poly(ADP-ribose) metabolism in intact cells. To determine the extent of PARP activation during apoptosis and the relation to its cleavage, HL60 cells were treated with the topoisomerase II inhibitor, VP-16. PARP cleavage occurred 3 h after treatment, concomitantly with a transient polymer synthesis (Fig. 1). The 89-kDa apoptotic fragment of PARP was poly(ADP-ribose)ylated as recognized by the antiseraum for PARP, whereas full-length PARP was not (Fig. 1B).

**An immunological method recently developed in our laboratory** (20) was then used to directly measure the endogenous polymer levels. This technique relies on affinity chromatography purification of the polymers followed by their specific immunodetection. PARP cleavage was measured in parallel using Western blotting with C11-10 antibody. The time course experi-
DNA ladder appeared at the same time point as the poly(ADP-ribose) peak, strongly suggesting that PARP activation was caused by these strand breaks.

Poly(ADP-ribosyl)ation of PARP Stimulates Its Cleavage by Caspase-7, but Not by Caspase-3—The results obtained from the apoptotic cells suggested that PARP cleavage occurs in vivo when the enzyme is poly(ADP-ribosyl)ated in response to DNA strand breaks. To define more precisely the effect of PARP automodification on caspase activity, we investigated the in vitro kinetics of automodified PARP cleavage by purified caspase-3 and -7. PARP was automodified in the presence of NAD and activated DNA and then subjected to cleavage assay by caspase-3 and -7. Because highly modified PARP cannot be resolved by gel electrophoresis (23), poly(ADP-ribose) glycohydrolase, the enzyme responsible for poly(ADP-ribose) catabolism, was added to the cleaved poly(ADP-ribosyl)ated PARP to remove the polymers that impair its mobility on gel. Under these conditions, almost all of the polymers could be removed (data not shown), ensuring a precise determination of the PARP cleavage kinetics. As control, PARP was digested under the same conditions except that the NAD and DNA were omitted, because the binding of PARP on DNA delays its cleavage (25). Because the specific activity may vary between enzyme preparations, the same enzymatic activity was used for each caspase. The results shown in Fig. 3 indicates that caspase-7 cleaves PARP with a greater efficiency than caspase-3. Furthermore, they demonstrate that PARP automodification greatly stimulates its proteolytic cleavage by caspase-7 but not by caspase-3. Using higher amounts of caspase-3, PARP was cleaved efficiently independently of its state of automodification (data not shown). Thus, PARP cleavage by caspase-7 but not by caspase-3 is specifically increased by the poly(ADP-ribosyl)ated substrate. The effect of free poly(ADP-ribose) on caspase activity was also measured, using the synthetic substrate DEVD-pNA to ensure that the polymers would not interact with the caspase substrate. The presence of 10 μM free poly(ADP-ribose) had no effect either on caspase-3 or on caspase-7 activity (data not shown). Thus, the stimulation of PARP cleavage by caspase-7 is not mediated by a direct effect of the free polymer on caspases. Other experiments showed similar caspase activity in the absence or in the presence of NAD, activated DNA and DHQ (data not shown). None of these compounds had an effect on caspase activity at the concentrations found in the PARP automodification reaction mixture.
Caspase-7 Cleavage of Poly(ADP-ribosyl)ated PARP

To determine the length of poly(ADP-ribose) that stimulates caspase-7, PARP was automodified in presence of DNA and various concentrations of NAD, after which the automodified substrate was digested with caspase-7 for 20 min. Automodification with polymers up to 20 residues had only minimal effect on caspase-7 activity (Fig. 4, A and B). The presence of DNA, which has been shown to delay PARP cleavage by caspase-3 (25), could explain the absence of cleavage at concentrations of NAD below 0.1 μM. However, PARP automodification with the long and branched polymers produced at 200 μM NAD resulted in a strong stimulation of caspase-7 (Fig. 4, A–C). The length of the polymers was then measured in the apoptotic cells using a new technique consisting in the separation of the polymers on a polyacrylamide gel and autoradiographed (B). Poly(ADP-ribose) produced in vitro at 200 μM NAD (C) or purified from HL60 cells treated for 3 h with 68 μM VP-16 (D) were also transferred on a Hybond N membrane after polyacrylamide gel electrophoresis and revealed with LP96–10 antibody. Xylene cyanol and bromphenol blue migrate at poly(ADP-ribose) lengths of 20 and 8, respectively.

Evidence for in Vivo Cleavage of PARP by Caspase-7—Because our results strongly suggest that caspase-7 is responsible for the cleavage of activated PARP in vivo, we verified the activation of caspase-7 in HL60 cells in response to VP-16 treatment. As shown in Fig. 6A, caspase-7 was effectively processed to its active form in the apoptotic cells as seen by the appearance of its p19 subunit. This activation occurred 3 h after the treatment (data not shown), the time at which PARP was cleaved, suggesting that caspase-7 could effectively cleave PARP in vivo. Furthermore, there was active caspase-7 located in the nucleus, where it could cleave and inactivate poly(ADP-ribosyl)ated PARP (Fig. 6A). Caspase-7 was also activated at the time of PARP cleavage in MCF-7 cells treated with 1 μM staurosporine (Fig. 6, B and C). Because MCF-7 cells lack caspase-3 (32), these results further support that caspase-7 cleaves PARP in vivo.

DISCUSSION

During apoptosis, NAD depletion with partial restoration in the presence of PARP inhibitors has been reported (11, 29, 30). PARP activation has also been shown in the early (29, 33, 34) as well as in later stages of apoptosis (29, 35, 36). However, poly(ADP-ribose) levels resulting from the activation of PARP were measured using NAD incorporation in permeabilized cells. Major drawbacks of this technique are the inability to measure polymer levels precisely and the presence of artifactual high amounts of polymer, even in control cells (35). Thus, in this study, endogenous poly(ADP-ribose) was measured directly in intact HL60 cells to follow the activation of PARP during the course of VP-16-induced apoptosis. Using an immunological method for the quantification of polymer, we found that the activation of PARP coincided with the appearance of the DNA ladder. Furthermore, this endogenous polymer peak was sharp when compared with the one found using permeabilized cells (35, 36), suggesting that PARP was activated only at the time of the appearance of the DNA ladder and not before. This suggests an activation of PARP by DNA strand breaks generated during internucleosomal DNA degradation, as proposed by Nosseri et al. (29). Activation of PARP in response to apoptosis inducers like anti-Fas antibody or camptothecin has also been shown by Western blot using an antibody against poly(ADP-ribose) (34).

During the course of VP-16-induced apoptosis of HL60 cells, PARP activation was low compared with the amount of DNA damage: 50 μM of the alkylating agent 1-methyl-3-nitro-1-nitroso-2-guanidine (MNNG) caused complete PARP cleavage in these cells. Using 0.1 μM MNNG was used to determine caspase-3 and -7 affinities for poly(ADP-ribose). As shown in Fig. 4A, the binding of [32P]poly(ADP-ribose) (A, 0.1 μM; B, 1 μM) or 6.58 μM [32P]-DNA (C) membranes were washed, and the radioactive polymer (or DNA) still bound to the membrane was analyzed by autoradiography.
that caspase-7 was more effective in cleaving PARP than HL60 cells treated for 4 h with 68 µM staurosporine for the indicated times (B) were resolved on a 15% polyacrylamide gel and submitted to Western blotting using a polyclonal antibody against caspase-7 p19. PARP cleavage was also visualized in the MCF-7 cells using CII10 antibody (C). PARP cleavage was observed in caspase-3 knockout mice (3, 5). PARP was also cleaved in the caspase-3 defective cell line MCF-7, cleavage that paralleled caspase-7 activation (Fig. 6, B and C). These results all lend support to the notion that caspase-7 is the most efficient caspase for PARP cleavage. They also indicate that the tetrapeptide cleavage site is not the only important factor for efficient cleavage of caspase substrates. Furthermore, the poly(ADP-ribosyl)ation of PARP appears to target caspase-7 to the activated PARP to ensure its rapid inactivation.

To maintain cellular integrity and homeostasis, a set of proteins act as stress sensors to promote a cellular response to situations such as DNA damage or heat shock. Because apoptosis results in the complete dismantling of the cell, such stress signaling proteins may be stimulated and interfere with the apoptotic process. However, this could be prevented by the rapid inactivation of these proteins by caspases. Because DNA is rapidly degraded in apoptotic cells, inhibition of DNA repair is primordial in this view. Because PARP can act as a DNA break sensor to signal DNA damage, the rapid cleavage of the activated PARP molecules would ensure the interruption of this signal. Interestingly, similar reasoning could apply to another DNA break-sensing molecule, DNA protein kinase. It has been reported that the active DNA protein kinase holoenzyme, bound to DNA breaks, would be the physiological target of caspase-3 (41). Such a preferential cleavage would permit a much more rapid execution phase of apoptosis, because only the active form of these stress-sensing proteins would need to be inactivated before dismantling of the cell, although all the molecules would eventually be cleaved independently on their state of activation. The failure to cleave those apoptotic substrates would not prevent the death of the cell but could retard it sufficiently to render it dangerous for the organism. Such an inhibition of apoptotic morphology has recently been shown for PARP (42).

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