Role of Poly(ADP-ribose) Polymerase (PARP) Cleavage in Apoptosis

CASPASE 3-RESISTANT PARP MUTANT INCREASES RATES OF APOPTOSIS IN TRANSFECTED CELLS*

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An early transient burst of poly(ADP-ribosyl)ation of nuclear proteins was recently shown to be required for apoptosis to proceed in various cell lines (Simbulan-Rosenthal, C., Rosenthal, D., Iyer, S., Boulares, H., and Smulson, M. (1998) J. Biol. Chem. 273, 13703–13712) followed by cleavage of poly(ADP-ribose) polymerase (PARP), catalyzed by caspase-3. This inactivation of PARP has been proposed to prevent depletion of NAD (a PARP substrate) and ATP, which are thought to be required for later events in apoptosis. The role of PARP cleavage in apoptosis has now been investigated in human osteosarcoma cells and PARP −/− fibroblasts stably transfected with a vector encoding a caspase-3-resistant PARP mutant. Expression of this mutant PARP increased the rate of staurosporine and tumor necrosis factor-α-induced apoptosis, at least in part by reducing the time interval required for the onset of caspase-3 activation and internucleosomal DNA fragmentation, as well as the generation of 50-kilobase pair DNA breaks, thought to be associated with early chromatin unfolding. Overexpression of wild-type PARP in osteosarcoma cells also accelerated the apoptotic process, although not to the same extent as that apparent in cells expressing the mutant PARP. These effects of the mutant and wild-type enzymes might be due to the early and transient poly(ADP-ribose) synthesis in response to DNA breaks, and the accompanying depletion of NAD apparent in the transfected cells. The accelerated NAD depletion did not seem to interfere with the later stages of apoptosis. These results indicate that PARP activation and subsequent cleavage have active and complex roles in apoptosis.

Apoptosis plays important roles in development, immunological competence, and homeostasis. It is characterized by marked changes in cellular morphology, including chromatin condensation, membrane blebbing, nuclear breakdown, and the appearance of membrane-associated apoptotic bodies, internucleosomal DNA fragmentation, as well as by cleavage of poly(ADP-ribose) polymerase (PARP).1 PARP catalyzes the poly(ADP-ribosyl)ation of a variety of nuclear proteins with NAD as substrate. Because it is activated by binding to DNA ends or strand breaks, PARP was suggested to contribute to cell death by depleting the cell of NAD and ATP (1, 2). PARP was subsequently shown to be cleaved into 89- and 24-kDa fragments that contain the active site and the DNA-binding domain of the enzyme, respectively, during drug-induced apoptosis in a variety of cells (3–5). Such cleavage essentially inactivates the enzyme by destroying its ability to respond to DNA strand breaks.

Caspase-3, a member of the caspase family of 13 aspartate-specific cysteine proteases that play a central role in the execution of the apoptotic program (6–8), is primarily responsible for the cleavage of PARP during cell death (4, 5, 9). The sequence at which caspase 3 cleaves PARP (DEVD) is very well conserved in the PARP protein from very distant species, indicating the potential importance of PARP cleavage in apoptosis (9). In human osteosarcoma cells that undergo spontaneous, confluence-associated apoptosis over a 10-day period in culture, caspase-3-like activity measured with a specific [35S]PARP-cleavage assay in vitro peaks at 6–7 days after initiation of apoptosis, concomitant with the onset of internucleosomal DNA fragmentation (4). We have also previously examined the time course of PARP activation during spontaneous apoptosis in these cells by immunofluorescence microscopy with antibodies to poly(ADP-ribose) (PAR) (10). A transient burst of PARP synthesis from NAD was observed that increased early and peaked 3 days after initiation of apoptosis, prior to the appearance of internucleosomal DNA cleavage. We have recently detected such a transient poly(ADP-ribose)lation of nuclear proteins at an early stage of induced apoptosis in various other cell types (11, 12). Prevention of this early poly(ADP-ribose)lation, either by disruption of the PARP gene or by expression of PARP antisense RNA, prevented completion of the apoptotic program as assessed on the basis of various morphological and biochemical markers of cell death.

Several strains of PARP knockout mice have been established (13, 14). Despite variations in physiological phenotype among these animals, they are viable and developmentally normal. Mitochondrial apoptosis appears to occur in the absence of PARP (15). However, “abnormal” apoptosis has been described in some of the knockout animals (9). As mentioned above, we have shown that fibroblasts derived from one strain of PARP knockout mice fail to show either the early burst of poly(ADP-ribose)lation or subsequent markers of apoptosis when exposed to agents that induce apoptotic death in fibroblasts from control mice (11, 12).

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‡ The abbreviations used are: PARP, poly(ADP-ribose) polymerase; PAR, poly(ADP-ribose) substrate; ATP, adenosine triphosphate; TNF, tumor necrosis factor; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair(s).

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Our previous studies with clonal cells depleted of endogenous PARP by expression of PARP antisense RNA have also revealed that this enzyme plays important roles in genomic stability (16), adipocyte differentiation (17), DNA replication associated with this differentiation (12, 18), and DNA repair (19).

To investigate the role of PARP degradation in the later stages of apoptosis, we have now expressed a mutant PARP that is resistant to cleavage by caspase-3 in human osteosarcoma cells and PARP →/− fibroblasts and examined its effect on both staurosporine and TNF-α-induced apoptosis in these cells. We also monitored the fate of the both the endogenous and recombinant PARP molecules as well as the concentration of NAD in the transfected cells.

**EXPERIMENTAL PROCEDURES**

*Construction of Mutant PARP cDNA*—A cDNA encoding a caspase-3-resistant form of PARP was generated with the use of a QuickChange site-directed mutagenesis kit (Stratagene). The resistance-conferring mutation was introduced with the use of two 37-base complementary oligonucleotides (5′-GGCGGCGGC-AAGTCGTCGTGCTCACTTGTCATCGTCGTGCTGCGTTTCCCTCACTCCATCCTCCACCTACGGGAGGTCTTAAAATTG-3′ and 5′-GAAAAAGCGATGGGTTGGGAGTTGATAGAAGTTGGC-3′) whose sequences match those of human wild-type PARP cDNA (20) with the exception that they encode glycine, rather than arginine, at 214 of the PARP-encoding cDNA sequence. After a 20-min annealing reaction at 65 °C, the oligonucleotides were subjected to an enzymatic reaction that resulted in ligation, at position 214 (bold sequence). Both wild-type and mutant PARP cDNA were amplified by polymerase chain reaction with Pfu DNA polymerase (Stratagene) according to recommendations of the manufacturer. The amplification primers (sense, 5′-GGCGGCGGC-GACAGGAGGTCTTAAAATTG-3′ and antisense, 5′-GGCGGCGGC-AGTTGGGAGTTGATAGAAGTTGGC-3′) introduced an NheI restriction site into the 5′ end and a NotI restriction site into the 3′ end of the PARP cDNA. A pair of oligonucleotides encoding multiple histidine residues and the FLAG epitope (5′-GGGGGCGGCC-ACAGGAGGTCTTAAAATTG-3′ and 5′-CTAGTCACTTGTCATCGTCGTGCTGCGTTTCCCTCACTCCATCCTCCACCTACGGGAGGTCTTAAAATTG-3′) were annealed to the 5′ and 3′ ends of the transfection plasmid respectively to create a 37-base complementary oligonucleotide fragment. The resultant 5′ and 3′-oligonucleotides were ligated into the 3′ NotI and 5′ XbaI restriction sites of the pcR3.1 (Invitrogen) that had been digested with NheI and XbaI.

*Assay of PARP Cleavage and Activity*—Plasmids encoding wild-type or mutant PARP were used to synthesize [35S]methionine-labeled proteins by coupled T7 RNA polymerase-mediated transcription and translation in a reticulocyte lysate (Promega). For assay of PARP cleavage, portions (1 μg) of lysate containing described transfected or nontransfected cells were incubated for 30 min at 37 °C in the absence or presence of 10 ng of caspase-3 (kindly provided by D. Nicholson, Merck). The reaction was stopped by the addition of SDS sample buffer, and the products were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. For assay of PARP activity, 5 μl of lysate containing recombinant protein were incubated with sonicated salmon sperm DNA and [32P]NAD (NEN Life Science Products) as described previously (21).

*Cell Culture, Transfection, and Induction of Apoptosis*—Human osteosarcoma cells (American Type Culture Collection) and PARP (Roche Molecular Biochemicals), to CPP32 (kindly provided by D. Nicholson, Merck), to the FLAG epitope (Santa Cruz Biotechnology), or to PAR (10H-A) (11). Immune complexes were detected with appropriate secondary antibodies and chemiluminescent reagents (Pierce).

*Assay of Caspase-3 Activity*—Caspase-3 activity was measured essentially as described (23). In brief, extracts (25 μg of protein) were incubated for 30 min at 37 °C with 40 μM DEVD-aminomethylcoumarin (AMC) peptide substrate in a total volume of 200 μl. The fluorescence of free AMC, generated as a result of cleavage of the aspartate-AMC bond, was monitored continuously over 15 min with a CytoFluor 4000 fluorometer at excitation and emission wavelengths of 360 and 460 nm, respectively. The emission from each sample was corrected against linear regression analysis of the initial velocity (slope) for each curve yielded the activity.

*Assay of NAD*—Cells were seeded into six-well culture dishes at a density of 1 × 10⁶ cells/well. After treatment with staurosporine for various times, the cells were harvested and washed with cold PBS. The cell pellet was then resuspended in 100 μl of lysis buffer (100 mM EDTA (pH 8.0), 20 mM NaCl, 10 mM Tris (pH 8.0)), mixed with 150 μl of agarose solution (1% agarose in lysis buffer, kept at 42 °C) and poured into a plug mold. After solidification, plugs were washed with 10 ml of lysis buffer supplemented with 1 mg/ml protease K and 1% sodium lauryl sarcosinate at 50 °C. Plugs were then incubated for at least 4 h in 1 ml of Tris-EDTA buffer (pH 7.4) at 4 °C with at least two changes of buffer and then stored at 4 °C until usage. DNA was subjected to a 1% agarose in 1× PFE buffer (20× PFE, 0.2× Tris, 7.85 mM EDTA, and 0.5% glacial acetic acid) at 170 V for 30 min with 4-s pulses followed by 150 V for 18 h with 35-s pulses. This program allowed for resolution of DNA molecules up to 1000 kb. Lambda DNA ladders (50–1000 kb) were used.
as standards. DNA fragments were visualized by staining with ethidium bromide.

RESULTS

Induction of Apoptosis in Osteosarcoma Cells by Staurosporine—We have previously used a human osteosarcoma cell line to identify the enzyme (caspase-3, originally termed apopain) responsible for PARP degradation during apoptosis as well as to study the role of PARP in the death program in individual cells by immunofluorescence microscopy (4, 10). These cells undergo a slow spontaneous apoptosis related to confluence. We therefore investigated the effects of expression of a caspase-3-resistant PARP mutant on apoptosis in these cells.

In addition to confluence-associated apoptosis, the osteosarcoma cells undergo apoptosis on exposure to the protein kinase inhibitor staurosporine. Treatment of the cells with 1 μM staurosporine for 24 h reduced cell viability to 40% of control values, as assessed by measuring calcein fluorescence (Fig. 1A). Staining of the cells with Hoechst 33342 revealed that staurosporine induced chromatin condensation and the typical morphological changes characteristic of apoptosis (Fig. 1B). Agarose gel electrophoresis also showed that treatment of cells with staurosporine for 24 h induced internucleosomal DNA fragmentation (Fig. 1C).

Construction and Characterization of Caspase-3-resistant PARP—We have previously shown that PARP is cleaved between Asp214 and Gly215 (20) by caspase-3 (4). To investigate the role of PARP and its cleavage in apoptosis, we constructed a cDNA encoding a mutant PARP (mut-PARP) in which Asp214 is replaced by Gly (Fig. 2A). This substitution theoretically renders the protein resistant to cleavage by caspase-3. This construct as well as a cDNA encoding wild-type PARP (wt-PARP) were separately introduced into an expression vector together with a nucleotide sequence encoding the FLAG epitope.

To demonstrate that the protein produced from the mut-PARP cDNA was both catalytically active and resistant to caspase-3 cleavage, we first performed several in vitro experiments. Complementary DNAs encoding mut-PARP or wt-PARP, with or without the FLAG sequence, were transcribed and translated with a reticulocyte lysate. For assay of susceptibility to caspase-3, the in vitro translated proteins labeled with [35S]methionine were incubated with the recombinant protease and the reaction products were analyzed by SDS-PAGE and autoradiography. Whereas wt-PARP was cleaved into its characteristic 89- and 24-kDa fragments on exposure to caspase-3, mut-PARP was not (Fig. 2B). The presence of the FLAG sequence did not affect this action (or lack thereof) of caspase-3 on wt-PARP (or mut-PARP). Although caspase-3 did not cleave mut-PARP into 89- and 24-kDa fragments, it appeared to cleave a small fragment from the mutant protein, so that it migrated as a doublet after caspase-3 treatment; this observation suggests the presence of a cryptic second cleavage site for caspase-3. The rate of cleavage at this second site was much slower than that at position 214 (data not shown). Similar results, including cleavage of mut-PARP at the second site, were obtained when [35S]methionine-labeled mut-PARP and wt-PARP were incubated with extracts prepared from Jurkat cells undergoing apoptosis induced by antibodies to Fas (data not shown).

The activity of the in vitro translated FLAG-tagged PARP proteins was measured by incubating reticulocyte lysate with [32P]NAD and fragmented DNA and then measuring incorporation of radioactivity into proteins precipitated with trichloroacetic acid. Both wt-PARP and mut-PARP exhibited substantial poly(ADP-ribosyl)ation activity (Fig. 2C), and this activity was blocked by the PARP inhibitor 3-aminobenzamide. We also examined the effect of caspase-3 treatment on the activity of both wt- and mut-PARP. Reticulocyte lysates containing either the mutant or wild-type enzyme were incubated with recombinant caspase-3, under conditions sufficient to result in cleavage of

![Fig. 1. Induction of apoptosis in osteosarcoma cells by staurosporine.](image-url)
of a small fragment from the mutant protein (PARP proteins, or lysate alone, were incubated for 10 min with sonicated DNA and [32P]NAD in the absence or presence of 3-aminobenzamide.

Construction and characterization of mut-PARP in vitro. A, PARP contains three major domains: a DNA-binding domain containing two zinc fingers, an automodification domain, and a catalytic domain. Cleavage of wild-type PARP (wt-PARP) between Asp214 and Gly215 produced a mutant PARP (mut-PARP) that is resistant to cleavage by caspase-3. Expression vectors encoding wt-PARP and mut-PARP, each fused to the FLAG epitope, were constructed. Both mut-PARP and wt-PARP, with or without the FLAG sequence, were synthesized by coupled transcription and translation with a reticulocyte lysate.

From the available text, we can infer that the diagram illustrates the domains of PARP and the sites of cleavage by caspase-3. The text also describes the synthesis and characterization of wild-type and mutant PARP proteins and their resistance to cleavage by caspase-3.

Stable Transfection of Osteosarcoma Cells with PARP Vectors and Fate of wt- and mut-PARP during Staurosporine-induced Apoptosis—Osteosarcoma cells were stably transfected with vectors encoding either wt- or mut-PARP, and individual clones were isolated by G418 selection and shown to express the recombinant proteins by immunoblot analysis of cell extracts with antibodies to FLAG (Fig. 3A). Although Western analysis is not strictly quantitative, the expression of wt-PARP appeared to be about 2-fold greater than that of mut-PARP. Since the mutation introduced to PARP is located in the nucleolar localization sequence of the protein, we wished to determine whether it had an effect on the translocation of the mutant protein into the nucleus of transfected cells. Indirect immunostaining with anti-FLAG antibodies revealed no difference in nuclear translocation between wt- and mut-PARP (Fig. 3B), indicating that the mutation did not interfere with mut-PARP translocation. The fate of endogenous PARP in nontransfected cells and that of the recombinant PARP proteins in transfected cells during staurosporine-induced apoptosis were monitored by immunoblot analysis with antibodies to PARP or to FLAG, respectively. Both endogenous PARP (Fig. 3C) and wt-PARP (Fig. 3D) were apparently cleaved by caspase-3, as revealed by the generation of the 89-kDa fragment (the 24-kDa fragment is not shown because both anti-PARP and anti-FLAG antibodies recognize only the COOH termini of the respective proteins). Cleavage of wt-PARP was detected at an earlier time point (3 h) than was that of endogenous PARP (6 h), and it proceeded at a relatively constant rate until 24 h, when most of the intact protein had disappeared. In contrast, mut-PARP was not cleaved to produce the 89-kDa fragment during the first 12 h of exposure to staurosporine (Fig. 3E), indicating that the protein was resistant to the action of caspase-3 in intact cells as well as in vitro. However, at 24 h, mut-PARP was degraded, although not to the 89-kDa fragment. Similar degradation was observed when [35S]methionine-labeled mut-PARP was incubated in vitro with extracts of Jurkat cells at late stages of apoptosis induced by antibodies to Fas (data not shown). This degradation of mut-PARP is thus likely the result of the non-specific proteolysis activity that occurs in late stages of apoptosis.

Effects of Expression of mut-PARP on Cell Viability and DNA Fragmentation in Staurosporine-treated Cells—The effects of wt-PARP and mut-PARP on the time course of the decrease in cell viability during staurosporine-induced apoptosis were assessed by measurement of calcein fluorescence. In nontransfected cells, staurosporine reduced viability by only 18% at 12 h and by 60% at 24 h (Fig. 4A), with 80–90% of cells having died by 36 h (data not shown). Transfection of these cells with an empty vector did not change their sensitivity to staurosporine (data not shown). The expression of mut-PARP greatly increased the rate of cell death; a decrease in viability was apparent as early as 3 h, by 6 h more than 30% of cells were nonviable, and by 24 h more than 90% of cells had died. The rate of cell death was also increased in cells expressing wt-
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Fig. 3. Fate of endogenous PARP and of wt- and mut-PARP during staurosporine-induced apoptosis in osteosarcoma cells. A, levels of expression of wt- and mut-PARP in transfected cells. Whole-cell extracts were prepared and equal quantities of total protein were subjected to immunoblot analysis with antibodies to the FLAG epitope. B, nuclear translocation of mut-PARP in transfected cells. Cells were fixed and incubated with anti-FLAG antibodies followed by incubated Cy 3-conjugated anti-mouse antibodies and costained with Hoechst 33342, then analyzed by fluorescence microscopy. Nontransfected cells (C) and cells transfected with vectors encoding either wt-PARP (D) or mut-PARP (E) were incubated for the indicated times with 1 μM staurosporine to induce apoptosis. Whole-cell extracts were then prepared and subjected to immunoblot analysis with antibodies either to PARP (C) or to the FLAG epitope (D and E). The positions of the 116-kDa intact PARP protein and the 89-kDa cleavage product are indicated on the right.

PARP, although not to as great an extent as in cells expressing mut-PARP.

We next examined the effects of wt- and mut-PARP on staurosporine-induced DNA fragmentation. Whereas internucleosomal DNA fragmentation was not apparent until 24 h after exposure to staurosporine in nontransfected cells, this fragmentation was first detected at 3 h in cells expressing either wt- or mut-PARP (Fig. 4B); the extent of DNA fragmentation at 3 h was slightly, but consistently noted to be greater in cells expressing mut-PARP than in those expressing wt-PARP. The occurrence of internucleosomal DNA fragmentation in the transfected cells indicates that, as in the control cells, staurosporine-induced death is mediated by apoptosis. Similar results were obtained with Jurkat cells that were transfected with the same constructs and in which apoptosis was induced by antibodies to Fas or etoposide (data not shown).

Effect of mut-PARP on Caspase-3 Activation—Caspase-3 activation has been shown to be required for DNA fragmentation (25, 26). We therefore examined whether the early onset of DNA fragmentation in cells expressing wt- or mut-PARP was accompanied by activation of caspase-3 at earlier time points. Extracts prepared from cells incubated with staurosporine for various times were assayed for caspase-3 activity by a fluorescence-based assay with a synthetic substrate. Expression of mut-PARP indeed shifted the time course of caspase-3 activation to earlier time points (Fig. 5A). Thus, in contrast to nontransfected cells, cells expressing mut-PARP showed a marked increase in caspase-3 activity as early as 3 h after exposure to staurosporine, consistent with the DNA fragmentation observed at this time (Fig. 4B). Cells overexpressing wt-PARP also showed an earlier activation of caspase-3 (Fig. 5A), which correlated with the earlier cleavage of PARP (Fig. 3B) and earlier DNA fragmentation (Fig. 4B) apparent in these cells. These results were confirmed by immunoblot analysis with antibodies to the caspase-3 precursor CPP32, which also recognize the p17 subunit of caspase-3 and a 20-kDa processing intermediate (p20) (Fig. 5B).

Effect of mut-PARP on Cleavage of Endogenous PARP during Apoptosis—To investigate whether the earlier onset of caspase-3 activation in cells expressing wt- or mut-PARP was reflected in an earlier cleavage of endogenous PARP, we reprobed the nitrocellulose filters from the experiments shown in Fig. 5A with antibodies to PARP. The cleavage of endogenous PARP in cells expressing wt-PARP or mut-PARP was first apparent 3 h after exposure to staurosporine (Fig. 5C), in contrast to the 6-h exposure required to detect such cleavage in nontransfected cells (Fig. 3A) and correlating with the earlier onset of caspase-3 activation. The time course of cleavage of wt-PARP (Fig. 5C) was similar to that of cleavage of endogenous PARP in the same cells, indicating that the presence of the FLAG sequence in wt-PARP did not affect the kinetics of cleavage by caspase-3. Furthermore, these results show that the addition of the FLAG sequence rendered the proteins unrecognizable by the anti-PARP antibody (compare Fig. 3C with Fig. 5C).

Effect of mut-PARP on Poly(ADP)ribosylation—Because expression of wt- or mut-PARP resulted in an earlier onset of DNA fragmentation in staurosporine-treated cells, and because PARP activity is strictly dependent on binding of the enzyme to DNA ends or strand breaks (1), we next examined the kinetics of poly(ADP-ribosyl)ation in staurosporine-treated transfected and nontransfected cells. We have previously shown that osteosarcoma cells undergoing spontaneous apoptosis exhibit an early and transient burst of poly(ADP-ribosyl)ation of nuclear proteins shortly before the appearance of detectable internucleosomal DNA fragmentation (10, 11). In the present study, immunoblot analysis of cell extracts with antibodies to PAR revealed that poly(ADP-ribosyl)ation of PARP, which under these conditions was the predominant substrate for this modification (11, 12), was evident after exposure of nontransfected cells to staurosporine for 12 h (Fig. 6A), a time when ~50% of

A. Yakovlev, A. H. Boulares, S. Iyer, X. Wang, and M. Smulson, submitted for publication.
PARP had been cleaved by caspase-3 (Fig. 3A). In contrast, poly(ADP-ribosyl)ation of PARP was detected as early as 3 h after exposure of cells expressing mut-PARP to staurosporine (Fig. 6C). This earlier activation of PARP was consistent with the early appearance of DNA fragmentation in these cells (Fig. 4B). Similar results were obtained with cells expressing wt-PARP (Fig. 6B), although the rate of increase in PARP activity appeared less than that observed in cells expressing mut-PARP. These results indicate that the kinetics of PARP activation depend on both the amount of PARP available and the ability of caspase-3 to reduce that amount.

**Effect of mut-PARP on Intracellular NAD Concentration**—PARP activation and the accompanying extensive synthesis of PARP that occur in response to the DNA strand breakage induced by alkylating agents or other DNA-damaging agents have been shown to result in depletion of intracellular NAD, which Berger et al. (2, 27) have suggested is responsible for the cell death that follows such treatment. We therefore investigated whether the early PAR synthesis observed in staurosporine-treated osteosarcoma cells expressing wt- or mut-PARP affects the intracellular concentration of NAD. Whereas the intracellular abundance of NAD in nontransfected cells remained largely unchanged during exposure to staurosporine for 6 h, that in cells expressing mut-PARP was reduced by 60% and 80% after treatment with this agent for 3 and 6 h, respectively (Fig. 6D). The concentration of NAD in cells expressing wt-PARP also decreased during exposure to staurosporine, but not to the extent observed in mut-PARP-expressing cells. Thus, the increased rate of staurosporine-induced death in cells expressing wt- or mut-PARP correlates with a marked depletion of intracellular NAD, and presumably of its precursor ATP, early during apoptosis.

**Effect of mut-PARP Expression on Apoptosis-dependent 50-kb DNA Break Generation after Staurosporine Treatment**—Generation of high molecular weight (50–300 kb) DNA breaks has been shown to precede the internucleosomal degradation (28, 29). These large DNA breaks are thought to represent a critical step for cell commitment to apoptosis (28), perhaps involving the unfolding of chromatin from nuclear matrix sites (30), and might constitute a key factor in the transient burst of poly(ADP) ribosylation. To explore this further, we examined the appearance of high molecular weight DNA fragments in staurosporine-treated cells harboring the different species of PARP by pulse field electrophoresis. High molecular weight fragments are detected by the appearance of 50-kb fragments (28, 29).
Cells were treated with staurosporine and incubated for different time intervals. After each time point, cells were collected and treated as described under "Experimental Procedures." mut-PARP expression indeed caused a much earlier generation of large breaks (Fig. 7C) compared with control cells (Fig. 7A). 50-kb DNA fragments were detected as early as 1 h after staurosporine treatment, while in control cells, they were not detected until 6 h after treatment supporting the earlier appearance of PARP cleavage activity in mut-PARP-transfected cells (Fig. 5). Cells overexpressing wt-PARP also showed an earlier generation of large breaks (Fig. 7B). These results are consistent with the early activation of PARP manifested by PAR synthesis (Fig. 6) as well as the appearance of DNA fragmentation (Fig. 4).

**Effects of Expression of mut-PARP on Cell Viability in TNF-α or Staurosporine-treated PARP −/− Fibroblasts**—To confirm the effect of mut-PARP expression on the kinetics of apoptosis and eliminate the potential interference of endogenous PARP in osteosarcoma cells, we examined the effect of mut-PARP expression in transfected PARP −/− fibroblasts. Cells were stably transfected with either wt- or mut-PARP cDNA, and individual clones were isolated by hygromycin selection and shown to express the recombinant proteins by immunoblot analysis of cell extracts with antibodies to human PARP (data not shown). Cell clones expressing comparable levels of mut- or wt-PARP were treated with TNF-α plus cycloheximide or staurosporine for different time intervals after which cell viability was assessed by calcein staining. Cells expressing mut-PARP consistently showed higher sensitivity to TNF-α treatment (observed in at least six independent experiments) than those expressing wt-PARP (Table 1). This difference was more pronounced after 12 h of treatment. A difference in sensitivity between mut- and wt-PARP was also detected in response to staurosporine treatment, and, although very reproducible, it was smaller than that observed with TNF-α plus cycloheximide. These results are consistent with those observed in mut-PARP-transfected osteosarcoma cells and again emphasize both the importance as well as the complexity of PARP cleavage during apoptosis.

**DISCUSSION**

In the present study, we have examined the role of PARP cleavage in apoptosis. PARP is cleaved by caspase-3 early during apoptosis in many different cell lines. The cleavage of PARP between Asp214 and Gly215 results in the 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 the recruitment of the catalytic domain to sites of DNA damage. This cleavage of PARP has been suggested to occur in order to prevent depletion of energy (NAD and ATP) that is thought to be required for later stages of apoptosis (2). It is also thought that PARP cleavage serves to prevent futile repair of DNA strand breaks during the apoptotic program. We reasoned that, if the role of PARP cleavage is to spare cells from the energy depletion that would result from excessive poly-(ADP-ribosyl)ation of nuclear proteins, then the presence of an active and uncleavable PARP might interfere with the onset of apoptosis. However, we have now shown that interference with PARP cleavage actually enhances apoptosis.

We constructed a mutant PARP that is not only resistant to cleavage by caspase-3, but also fully active in the presence of DNA strand breaks. Similar substitution of an aspartic acid residue by an alanine residue at the cleavage site of the retinoblastoma protein also conferred resistance to cleavage by a caspase-3-like protease (31). In vitro experiments showed that the mutation introduced at the cleavage site of PARP did not interfere with the catalytic activity of the protein or its translocation to the nucleus of transfected cells. Although treatment of mut-PARP with purified caspase-3 revealed the apparent presence of a second cleavage site, cleavage at this site did not affect enzyme activity. The 89-kDa fragment generated from the cleavage of wt-PARP by caspase-3 also showed similar pattern but only when the reaction was incubated for an extended time (data not shown). Whether this second site plays a role in the fate of PARP during normal growth or in apoptosis remains to be determined. The mut-PARP protein was shown to be active in intact cells by transfecting fibroblasts derived from PARP knockout mice with the expression construct and...
were treated with 1 µM pressin wt-PARP (numbers of control cells (see “Experimental Procedures”).

The difference was less pronounced in staurosporine-induced cell death. These results further demonstrate the role of PARP cleavage in the kinetics of apoptosis and eliminate the possible interference of endogenous PARP with mut-PARP in osteosarcoma cells. These results also reduce the possible competition to DNA breaks of the 24-kDa caspase-3 cleavage product with mut-PARP. Thus, we do not believe that there is evidence for a transdominant role for the 24-kDa PARP product during apoptosis in our system.

Intermucleosomal DNA fragmentation is a characteristic event in apoptotic death. Expression of mut-PARP increased the rate of staurosporine-induced internucleosomal DNA fragmentation in osteosarcoma cells, indicating that the transfected cells were undergoing death by apoptosis and that mut-PARP contributes to events leading to DNA fragmentation. Proteolytic cleavage and processing of CPP32 to caspase-3 are required for DNA fragmentation during apoptosis (35). The time course of staurosporine-induced caspase-3 activation was shifted to earlier times in osteosarcoma cells expressing mut-PARP, consistent with the earlier onset of DNA fragmentation, earlier cleavage of endogenous PARP, and faster death kinetics in these cells. MCF-7 cells fail to undergo DNA fragmentation on induction of apoptosis because they do not express an active caspase-3 (25, 26). Introduction of the caspase-3 gene into these cells reestablished their ability to fragment DNA after treatment with staurosporine or other apoptotic stimuli (25, 26). We have previously shown that inhibitors of caspase-3, such as the tetrapeptide AcDEVD-CHO, also block apoptosis (4).

DNA fragmentation is mediated, at least in part, by an endonuclease that is activated by caspase-3. The latent cytosolic endonuclease CAD (or DFF45) is actually activated as a result of caspase-3-catalyzed cleavage of the associated inhibitor ICAD (or DFF45), thus allowing the active enzyme to enter the nucleus and to degrade DNA (36–39). The expression of mut-PARP might have enhanced ICAD/DFF45 processing in our system, indicating that the trans-}

### Table I

|                        | 12 h  | 24 h  | 36 h  |
|------------------------|-------|-------|-------|
| **TNF-α + cycloheximide** |       |       |       |
| wt                     | 89 ± 5| 33 ± 7| 21 ± 3|
| mut                    | 58 ± 6| 42 ± 3| 34 ± 4|

Expression of mut-PARP greatly increased the rate of staurosporine-induced cell death in osteosarcoma cells. A similar, but less marked, effect was observed in cells expressing wt-PARP. This effect of mut-PARP thus cannot be attributable simply to the abundance of the recombinant protein, given that the amount of recombinant protein in cells expressing wt-PARP was greater than that in cells expressing mut-PARP (Fig. 3A). This is consistent with the fact that overexpression of PARP in rodent cells sensitized the cells to the effects of DNA-damaging agents (34). Conversely, we have previously shown that depletion of PARP from cells, either by expression of PARP antisense RNA or by PARP gene disruption, confers resistance to apoptosis (11), indicating a role for PARP and poly(ADP-ribose)ylation of nuclear proteins in the initiation of the apoptotic process. Our results demonstrate the role of PARP cleavage in the onset of apoptosis.

Similar increase in sensitivity was observed in mut-PARP-transfected PARP −/− fibroblasts when compared with cells expressing wt-PARP after TNF-α and staurosporine treatment. The difference was less pronounced in staurosporine-induced cell death. These results further demonstrate the role of PARP
Role of PARP Cleavage in Apoptosis

dant housekeeping protein, it has been thought that its activity is limited by the number of DNA strand breaks rather than by the number of enzyme molecules (42). We have now shown that the expression of an uncleavable PARP in osteosarcoma cells resulted in an earlier activation of PARP, as revealed by PAR synthesis and as compared with nontransfected cells, in cells undergoing staurosporine-induced apoptosis. Thus, PAR synthesis in mut-PARP-expressing cells was apparent after exposure to staurosporine for only 3 h, whereas the same level of PAR synthesis was not apparent in control cells until after 12 h of staurosporine treatment. Because PAR synthesis occurs only in the presence of DNA strand breaks, the earlier onset of PAR synthesis in mut-PARP-expressing cells must be due to an earlier generation of DNA strand breaks (9). Indeed, with the use of pulse field gel electrophoresis, we detected large (–50 kb) DNA fragments extremely early (1 h) in caspase-3-resistant mut-PARP-expressing osteosarcoma cells, whereas such fragments were not apparent in control cells until after 6 h of staurosporine treatment. The fact that such DNA fragmentation was detected before PAR synthesis might be due to a limitation in the sensitivity of the anti-PAR antibodies.

As mentioned above, it has been thought that PARP cleavage might serve to prevent depletion of NAD and ATP that are required for later stages of the apoptotic program. Expression of mut-PARP resulted in a marked decrease in the intracellular concentration of NAD in staurosporine-treated cells. Expression of wt-PARP induced a similar, but less pronounced, effect. It is important, however, to note that the accelerated NAD depletion did not interfere with the later stages of apoptosis. The presence of uncleavable PARP in cells undergoing staurosporine-induced apoptosis. Thus, PAR synthesis was not apparent in control cells until after 12 h of staurosporine treatment. Because PAR synthesis occurs only in the presence of DNA strand breaks, the earlier onset of PAR synthesis in mut-PARP-expressing cells must be due to an earlier generation of DNA strand breaks (9). Indeed, with the use of pulse field gel electrophoresis, we detected large (–50 kb) DNA fragments extremely early (1 h) in caspase-3-resistant mut-PARP-expressing osteosarcoma cells, whereas such fragments were not apparent in control cells until after 6 h of staurosporine treatment. The fact that such DNA fragmentation was detected before PAR synthesis might be due to a limitation in the sensitivity of the anti-PAR antibodies.

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