Gain-of-function of poly(ADP-ribose) polymerase-1 upon cleavage by apoptotic proteases: implications for apoptosis

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SUMMARY

Poly(ADP-ribosyl)ation is an important mechanism for the maintenance of genomic integrity in response to DNA damage. The enzyme responsible for poly(ADP-ribose) synthesis, poly(ADP-ribose) polymerase 1 (PARP-1), has been implicated in two distinct modes of cell death induced by DNA damage, namely apoptosis and necrosis. During the execution phase of apoptosis, PARP-1 is specifically proteolyzed by caspases to produce an N-terminal DNA-binding domain (DBD) and a C-terminal catalytic fragment. The functional consequence of this proteolytic event is not known. However, it has recently been shown that overactivation of full-length PARP-1 can result in energy depletion and necrosis in dying cells. Here, we investigate the molecular basis for the differential involvement of PARP-1 in these two types of cellular demise. We show that the C-terminal apoptotic fragment of PARP-1 loses its DNA-dependent catalytic activity upon cleavage with caspase 3. However, the N-terminal apoptotic fragment, retains a strong DNA-binding activity and totally inhibits the catalytic activity of uncleaved PARP-1. This dominant-negative behavior was confirmed and extended in cellular extracts where DNA repair was completely inhibited by nanomolar concentrations of the N-terminal fragment. Furthermore, overexpression of the apoptotic DBD in mouse fibroblast inhibits endogenous PARP-1 activity very efficiently in vivo, thereby confirming our biochemical observations. Taken together, these experiments indicate that the apoptotic DBD of PARP-1 acts cooperatively with the proteolytic inactivation of the enzyme to trans-inhibit NAD hydrolysis and to maintain the energy levels of the cell. These results are consistent with a model in which cleavage of PARP-1 promotes apoptosis by preventing DNA repair-induced survival and by blocking energy depletion-induced necrosis.

Key words: PARP-1, Transdominant, Apoptosis, Necrosis, DNA-binding domain

INTRODUCTION

The maintenance of genomic integrity is critically important for the prevention of cancer and other genetic diseases (Hanahan and Weinberg, 2000). Therefore, eukaryotic organisms have developed numerous mechanisms to protect themselves from DNA lesions. When DNA damage is induced at low levels, checkpoint proteins are activated and delay the progression through the cell cycle in order to provide time for DNA repair factors to fix the incurred damage (Dasika et al., 1999). When DNA damage is more important, some of these checkpoint proteins, such as p53, commit the damaged cells to an active process of cell death known as apoptosis (Giaccia and Kastan, 1998). During this process, proteases (caspases) are activated and cleave a number of death substrates that are believed to engage the cell in a cascade of energy-requiring events leading to an orderly demise (Earnshaw et al., 1999). Interestingly, it appears that moderate levels of DNA damage are not sufficient to result in cell death per se because, in the absence of p53, cells repair the damage and continue to proliferate without obvious consequences (Griffiths et al., 1997). However, high doses of DNA damage results in a passive and unorganized mode of cell death known as necrosis (Bonfoco et al., 1995; Majno and Joris, 1995). This process is associated with a rapid depletion of cellular energy, which is believed to prevent the execution of the apoptotic program (Eguchi et al., 1997; Leist et al., 1997). Interestingly, the inhibition of caspases induces a shift in the mode of cell death, from apoptosis to necrosis, that is similar to the one observed when the energy levels are artificially depleted in cells undergoing apoptosis (Hirsch et al., 1997; Lemaire et al., 1998; Vercammen et al., 1998). Therefore, it appears that the cleavage of at least some death substrates and the maintenance of energy levels are both essential for the execution of the apoptotic program.

Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme that catalyses the transfer of the ADP-ribose moiety of NAD^+ to a specific subset of nuclear substrates in response to DNA damage (D'Amours et al., 1999; Shall and de Murcia, 2000). PARP-1 is involved at several levels of the cellular

response to DNA damage. Indeed, PARP-1 is an important regulator of the DNA base excision repair (BER) pathway and is essential for the maintenance of genomic integrity and for survival in response to genotoxic insults (Ménissier-de Murcia et al., 1997; Trucco et al., 1998; d'Adda di Fagagna et al., 1999; Vodenicharov et al., 2000). Also, PARP-1 is specifically proteolysed by caspases to a 24 kDa DNA-binding domain (DBD) and to a 89 kDa catalytic fragment during the execution of the apoptotic program (Kaufmann et al., 1993; Casiano et al., 1996). Finally, it has been shown recently that the overactivation of PARP-1 is responsible for the energy depletion that causes necrosis in response to high levels of DNA damage (Berger, 1985; Heller et al., 1995; Eliason et al., 1997; Virag et al., 1998; Ha and Snyder, 1999). The early kinetics as well as the extent of PARP-1 cleavage observed in vivo during apoptosis suggest that this cleavage might be a decisive event during the progression to cellular demise (Kaufmann et al., 1993; Casiano et al., 1996). This view has gained momentum recently from studies of caspase-resistant PARP-1 mutants. Indeed, these experiments have shown that cells expressing uncleavable PARP-1 shift their mode of cell death from apoptosis to necrosis in the presence of moderate levels of DNA damage (Kim et al., 2000b). Furthermore, these cells appear to disengage themselves from apoptosis and show increased survival in the presence of low levels of DNA damage (Oliver et al., 1998; Halappanavar et al., 1999). Taken together, these results suggest that PARP-1 cleavage is a highly significant event that promotes apoptosis by preventing both necrosis and cell survival.

Despite this compelling evidence, little is known of the molecular significance and of the biochemical consequences of PARP-1 cleavage during cellular demise. Studies on the full-length DBD of PARP-1 suggest that its apoptotic counterpart could promote apoptosis by trans-inhibiting poly(ADP-ribose) synthesis in vivo (Schreiber et al., 1995). However, the actual molecular mechanism underlying this inhibition is not clear at the moment and remains to be defined biochemically under controlled conditions. Here, we show that the cleavage of PARP-1 by caspases causes its inactivation in a purified system. Furthermore, we see that this cleavage generates a gain-of-function in the apoptotic fragment containing the DBD of PARP-1, which results in a strong transdominant inhibition of poly(ADP-ribose) synthesis both in vitro and in vivo and in the total abrogation of DNA BER. This study shows for the first time at the biochemical level that the inhibition of PARP-1 activity observed in the presence of its DBD occurs directly by competition for the activating sites on DNA. Our results are consistent with a model in which PARP-1 promotes apoptosis by preventing DNA repair-induced survival and by blocking energy depletion-induced necrosis.

MATERIALS AND METHODS

PARP-1 proteolysis

For reconstitution of PARP-1 activity following cleavage, affinity purified PARP-1 (Zahradka and Ebisuzaki, 1984; see below) was digested for 6 hours with recombinant caspase 3 at a substrate:protease molar ratio of 2.5:1, as previously described (Orth et al., 1996). For the large scale purification of the apoptotic fragments, purified PARP-1 was digested for 8 hours at 37°C with caspase 3 at a substrate:protease molar ratio of 13:1. Caspase 3 was purified to homogeneity from bacteria overexpressing the human protease (Orth et al., 1996). When necessary, results were corrected for the presence of residual amounts of uncleaved PARP-1, as determined in the linear range of detection of western blotting. Automated microsequencing of the proteolysed samples confirmed that caspase 3 cleaved PARP-1 at the apoptotic cleavage site ($DEVD^{214}/G^{215}$, human numbering), as seen in vivo (Lazebnik et al., 1994).

Poly(ADP-ribosyl)ation assays

Poly(ADP-ribosyl)ation assays were carried out as described (Zahradka and Ebisuzaki, 1984) with the following modifications. For the reconstitution of PARP-1 activity following proteolysis, cleaved or uncleaved PARP-1 were added to a final concentration of 20 nM in the reaction medium and poly(ADP-ribosyl)ation was monitored during a period of 5 minutes in the presence of a saturating amount of activating DNA (36 μg/ml calf thymus DNA, Type XV, Sigma). To assess the transdominant activity of the 24 kDa fragment, uncleaved PARP-1 was added to a final concentration of 20 nM in the reaction medium simultaneously with different concentrations of the N-terminal apoptotic fragment, as described in the legend of Fig. 3. Since the DNA used in these assays was containing an undefined number of activating sites (strand-breaks), experiments were performed to determine the efficiency of the calf thymus DNA to activate PARP-1. Under our experimental conditions, 21 μg/ml calf thymus DNA (Type XV, Sigma) resulted in the activation of full-length PARP-1 to half of its maximal velocity and was used to evaluate the transdominant activity of the 24 kDa fragment (data not shown). This control was necessary to optimize the sensitivity of the transdominant assay since saturating amounts of activating sites could theoretically over-ride the inhibitory effect of transdominant inhibitors (Schreiber et al., 1995).

Purification of PARP-1 and of its apoptotic fragments

PARP-1 was purified to physical homogeneity from bovine thymus as described (Zahradka and Ebisuzaki, 1984). The final enzyme preparation had a specific activity of 1341 U/mg protein and was used as starting material for all subsequent experiments. After extensive cleavage of PARP-1 with caspase 3 (see above), three volumes of binding buffer were added to the reaction medium and the resulting mixture was submitted to DNA-cellulose chromatography (12×80 mm column), as described previously (Zahradka and Ebisuzaki, 1984). The apoptotic fragments of PARP-1 were purified to near homogeneity by this single-step affinity chromatography, as evaluated by silver staining of polyacrylamide gels containing the pooled fractions (Desnoyers et al., 1995). The concentrations of the purified N- and C-terminal fragments used in this report are 85 nM and 65 nM, respectively. The fragments were stored at –80°C and were stable for at least 9 months under these conditions.

DNA repair assay

DNA repair reactions were carried out as described (Satoh and Lindahl, 1992) in the presence of various concentrations of apoptotic fragments. Whole cell extracts where prepared from the normal lymphoblastoid cell line GM01953C as described previously (Satoh and Lindahl, 1992).

Plasmid construction, transfection and establishment of stable clones expressing the apoptotic 24 kDa DBD of PARP-1

The eukaryotic vector pMMTV-FgAp contains a 729 bp *Hin*d III cDNA fragment encoding the apoptotic DBD of human PARP-1 (residues 1-214) under the control of the dexamethasone-inducible MMTV promoter. The vector also contains the neomycin resistance gene for selection on G418-containing medium. The sequence of this plasmid was confirmed by automated sequencing. Mouse L cells were chosen for transfection experiments because of their capacity to express constitutively the glucocorticoid receptor, allowing activation

of MMTV promoter with dexamethasone. Cells were routinely grown in a humidified 5% CO_2 atmosphere at 37°C in Minimum Essential Medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Stable transfectants expressing the apoptotic DBD were generated by lipofection (Gibco-BRL) and by selection for G418 resistance. Stable clones were initially selected using 600 μg/ml G418 and were subsequently grown in the presence of 400 μg/ml G418. For the screening experiments, induction was done with 1 mM dexamethasone for 24 hours before harvesting and characterization by western blotting. Extracts from 100,000 cells were prepared and separated on a 10% SDS-polyacrylamide gel and further analyzed with the anti-PARP-1 monoclonal antibody F1-23 (Lamarre et al., 1988). The clone presenting the best combined profile of low basal and high induced expression level was LMFA6.

Polymer measurements

Poly(ADP-ribose) levels were measured on 10 cm^2 plates and quantified by immunodot blot as described (Affar et al., 1998).

RESULTS

Loss-of-function of the C-terminal apoptotic fragment of PARP-1 following cleavage with caspase 3

In a previous study, Lazebnik and collaborators have shown that apoptotic proteases recognize and cleave hPARP-1 at the sequence $DEVD^{214}/G^{215}$, which lies approximately in the middle of the DBD of the enzyme (Lazebnik et al., 1994). Consequently, the apoptotic fragment containing the catalytic domain of PARP-1 (89 kDa) loses a significant proportion of the DBD of the enzyme (which includes the zinc-fingers region) upon cleavage. Nevertheless, it is possible that this cleavage of PARP-1 does not significantly inactivate the enzyme because the remaining DNA-binding elements (helix-turn-helix motifs) of the 89 kDa fragment could still mediate substantial DNA-dependent catalytic activation. In order to precisely characterize the enzymatic consequences of PARP-1 cleavage during apoptosis, we evaluated the catalytic activity of PARP-1 following proteolysis with caspase 3. Fig. 1 shows that cleavage of PARP-1 with caspase 3 reduced the catalytic activity of the cleaved enzyme by ~95% at the earliest time point evaluated. This drastic reduction in the poly(ADP-ribosyl)ation potential of the C-terminal fragment of PARP-1 corresponds with a loss of sensitivity of the fragment to the presence of DNA breaks. Indeed, the catalytic activity of cleaved PARP-1 in the presence of activating DNA was similar to the activity of uncleaved PARP-1 observed in the absence of DNA (Fig. 1).

Studies by Kameshita and colleagues have previously shown that the catalytically inactive fragments of PARP-1 resulting from papain cleavage could re-associate and recover significant enzymatic activity (Kameshita et al., 1986). This precedent indicates that the apoptotic fragments of PARP-1 may re-associate following cleavage and still be proficient in DNA damage-induced poly(ADP-ribose) synthesis. Alternatively, they may also gain a new, previously uncharacterized function upon re-association. To address these points, we extended the duration of our poly(ADP-ribosyl)ation assay under conditions favoring the re-association of both catalytic and non-catalytic fragments of PARP-1, as previously defined (Kameshita et al., 1986). As shown in Fig. 1, we extended our poly(ADP-ribosyl)ation reactions until maximal levels of poly(ADP-ribose) formation could be obtained with both native and proteolysed enzymes. We can clearly see from Fig. 1 that this approach did not modify the inhibition of PARP-1 following caspase cleavage. A slight increase in the amount of poly(ADP-ribose) synthesized by the proteolysed enzyme was observed over the experimental time course. However, this still represents a very small fraction (<6.0% on average) of the poly(ADP-ribosyl)ation activity of uncleaved PARP-1 at all time points tested (Fig. 1). In addition, the poly(ADP-ribosyl)ation activity of the cleaved enzyme saturated very rapidly as shown by the appearance of a plateau in the amount of poly(ADP-ribose) synthesized after 2.5 minutes of reaction (Fig. 1). This is in contrast to the results obtained with the intact (full length) enzyme, which showed substantial poly(ADP-ribosyl)ation activity during the second half of the time course (Fig. 1). Importantly, fragments of PARP-1 smaller than the 89 kDa apoptotic fragment were previously shown to be poly(ADP-ribosyl)ated as efficiently as the native enzyme itself (Holtlund et al., 1983; Kameshita et al., 1986). This indicates that the inhibition we observed was not due to an inherent limitation in the ability of the 89 kDa fragment to be poly(ADP-ribosyl)ated.

Purification of the apoptotic fragments of PARP-1

The results of the proteolysis experiment described above clearly indicate that the activity of the 89 kDa fragment of PARP-1 is deficient and/or inhibited following the cleavage of the enzyme. The reason why it is not activated in the presence of DNA breaks might be that the remaining DNA-binding elements of the molecule are not sufficient to mediate productive interactions with DNA. Alternatively, or in addition, it is possible that the apoptotic DBD of PARP-1 could still bind strongly to DNA and act as a transdominant inhibitor of the 89 kDa catalytic fragment. To test these two possibilities and to

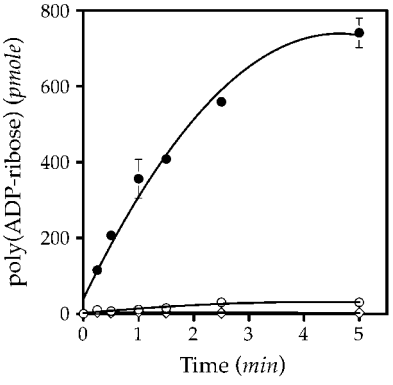

Fig. 1. Loss-of-function of PARP-1 following proteolysis by caspase 3. Native (●) and proteolysed (○) PARP-1 were compared for their poly(ADP-ribosyl)ation activity in the presence of saturating concentration of co-enzymic DNA over a period of 5 minutes. The activity of native PARP-1 was also measured in the absence of DNA (◇). Poly(ADP-ribose) synthesis was carried out until maximal level of product could be obtained for both the cleaved and intact enzyme in order to maximize the potential re-association of the apoptotic fragments. This approach was successfully used for the reconstitution of PARP-1 activity following limited proteolysis with papain (Kameshita et al., 1986). Assays were performed in triplicate and the s.d. for each time point is shown.

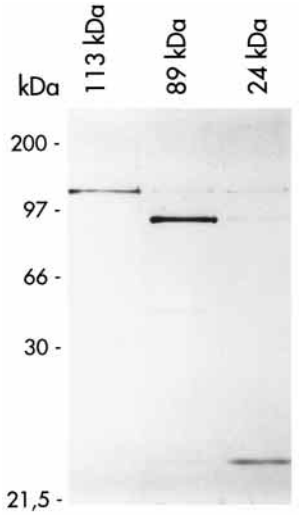

Fig. 2. Affinity purification of the apoptotic fragments of PARP-1. Bovine PARP-1 (purified to homogeneity) was cleaved with caspase 3 and subjected to ssDNA-cellulose affinity chromatography as described in Material and Methods. Silver-stained SDS-polyacrylamide gel (Desnoyers et al., 1995) showing the apoptotic fragments of PARP-1 obtained in late (~500 mM NaCl; 24 kDa) and early (89 kDa) fractions of the chromatography procedure.

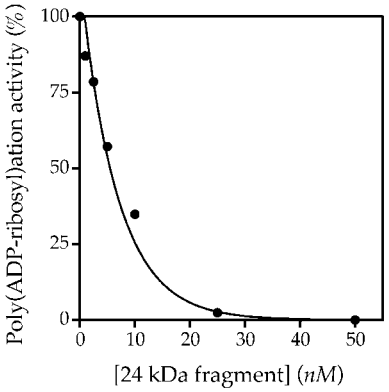

Fig. 3. Biochemical properties of the N-terminal apoptotic fragment of PARP-1. The catalytic activity of native PARP-1 was determined in the presence of increasing amounts of the 24 kDa apoptotic fragment of PARP-1. The results are expressed as percentage of maximal activity obtained in the absence of apoptotic fragment. Experiments were performed in triplicate at subsaturating concentrations of DNA strand-breaks, as described in Materials and Methods.

investigate the biochemical properties of the apoptotic fragments of the enzyme, we subjected purified PARP-1 to a semi-preparative-scale proteolysis with caspase 3 and subsequently compared the DNA-binding ability of the apoptotic fragments by ssDNA-cellulose chromatography. Our purification strategy was based on the observation that both fragments of the enzyme contain partial DBDs that are likely to show different DNA-binding properties. Accordingly, we were able to achieve a complete separation of the apoptotic fragments of PARP-1 by increasing linearly the ionic strength of the elution buffer during the chromatography (Fig. 2). Electrophoresis and silver staining of the pooled fractions from the chromatography indicated that the apoptotic fragments of PARP-1 were purified to near homogeneity by this approach (Fig. 2). Although showing substantial DNA-binding activity, the 89 kDa fragment eluted well before the 24 kDa zinc-finger-containing fragment of PARP-1, which remained tightly bound to the ssDNA matrix at NaCl concentrations up to ~0.5 M (data not shown). This chromatographic behavior is essentially similar to the previously reported elution profiles of the full-length DBD and native enzyme (Zahradka and Ebisuzaki, 1984; Kameshita et al., 1986). These results demonstrate that the N-terminal apoptotic fragment of PARP-1 retains the structural elements required for strong DNA-binding activity following cleavage by caspase 3. In addition, our results indicate that the 89 kDa fragment of PARP-1 is still able to bind DNA, although to a much lower extent than native PARP-1. This reduced affinity of the 89 kDa fragment for DNA, compared with intact PARP-1, is likely to contribute to the loss of poly(ADP-ribosyl)ation activity of the cleaved enzyme (Fig. 1).

Biochemical properties of the N-terminal apoptotic fragment of PARP-1

Since the N-terminal DNA-binding fragment of PARP-1 is physically separated from the automodification domain of the enzyme following caspase processing (Lazebnik et al., 1994), it is very likely that the dissociation constant of this fragment for DNA breaks will be greatly decreased under physiological conditions. To evaluate if binding of the 24 kDa fragment to DNA breaks could prevent DNA-binding and subsequent catalytic activation of the uncleaved enzyme, we performed poly(ADP-ribosyl)ation assays using full length PARP-1 in the presence of the 24 kDa fragment. These experiments were carried out in the presence of limiting concentrations of DNA breaks since saturating amounts of co-activating DNA could interfere with the potential transdominant activity of the 24 kDa fragment (Schreiber et al., 1995). As shown in Fig. 3, the addition of increasing amounts of the N-terminal fragment of PARP-1 in the poly(ADP-ribosyl)ation reaction decreased drastically the activity of native PARP-1, and resulted in complete inhibition at concentrations of ~35 nM and above. The inhibitory activity of the 24 kDa fragment was very potent as it showed an IC_{50} of 7 nM (Fig. 3). Since the concentration of uncleaved PARP-1 in the assay was 20 nM, these results indicate that complete inhibition of a PARP-1 molecule requires the binding of less than 2 molecules of the N-terminal apoptotic fragment to DNA breaks. This competitive inhibition of PARP-1 by its apoptotic (24 kDa) DBD is entirely consistent with the transdominant behavior of larger versions of the DBD of PARP-1 seen in vivo (Schreiber et al., 1995; Gaken et al., 1996). Furthermore, the kinetics of inhibition shown in Fig. 3 suggest that the apoptotic 24 kDa DBD might be a stronger inhibitor of PARP-1 activity than the 42 kDa DBD. Indeed, it has been shown by overexpression studies in mammalian cells that full inhibition of PARP-1 activity requires a tenfold molar excess of the 42 kDa DBD fragment relative to the endogenous enzyme (Schreiber et al., 1995). Taken together, these results indicate that the cleavage of PARP-1 leads to a new function of the 24 kDa fragment, which acts independently of the loss-of-(catalytic) function of the C-terminal 89 kDa fragment to inhibit poly(ADP-ribosyl)ation.

Transdominant inhibition of BER by the N-terminal apoptotic fragment of PARP-1

Since PARP-1 inhibitors negatively regulate DNA BER (Satoh and Lindahl, 1992), we next investigated if the transdominant activity of the 24 kDa fragment could also impinge on BER in a human cell-free assay. Fig. 4 illustrates the effect of increasing concentrations of the N-terminal fragment of PARP-1 on BER. In this assay, plasmids showing reduced electrophoretic mobility represent DNA substrates for BER (which contain one oxyradical-induced single-strand break/molecule), whereas the faster migrating form represents repaired plasmids (Satoh and Lindahl, 1992). Fig. 4A clearly shows that the presence of the apoptotic fragment of PARP-1 led to an important reduction in DNA repair at concentrations above 7 nM. DNA repair was strongly inhibited by concentrations of the N-terminal apoptotic domain ranging from 10 nM to 20 nM, and full inhibition of DNA BER was observed at a concentration of 28 nM (Fig. 4B). The C-terminal 89 kDa fragment of PARP-1 had no effect on DNA repair under these conditions (data not shown), which is consistent with the observation that this fragment is excluded from the nucleolus during apoptosis (Alvarez-Gonzalez et al., 1999). These results further establish that the 24 kDa apoptotic fragment of PARP-1 acquires a dominant-negative activity upon caspase processing. In addition, the results presented here strongly suggest that the effect of the 24 kDa fragment seen in the in vitro poly(ADP-ribosyl)ation system (Fig. 3) is a genuine effect as it can be extended to an inhibition of DNA repair in a more complex and representative environment (whole cell extract). Furthermore, the independent inhibition of both poly(ADP-ribosyl)ation and BER in the presence of saturating concentrations of NAD^+ indicates that the most probable mechanism of action of the 24 kDa fragment involves binding of the molecule to DNA break and steric inhibition of PARP-1 and other DNA repair factors.

In vivo activity of the apoptotic (24 kDa) DBD of PARP-1

Previous studies have demonstrated that the overexpression of the DBD of PARP-1 results in the inhibition of poly(ADP-ribose) synthesis and, consequently, in the inhibition of DNA BER in vivo. However, all these studies have used either fragments larger than the apoptotic DBD of PARP-1 or fusion (tagged) variants of the DBD. We have seen in the experiments described in Figs 3 and 4 that the kinetics of inhibition of both poly(ADP-ribose) synthesis and DNA repair differ significantly from the in vivo kinetics observed with the full-length DBD of PARP-1. These observations suggest that the size of the DBD influences its biological activity. To address this point and to evaluate the actual effect of the native, untagged version of the apoptotic DBD, we have generated a stable cell line expressing the apoptotic (24 kDa) DBD of human PARP-1. The expression of the DBD was induced by the addition of dexamethasone on the cells for 16 hours and resulted in strong levels of expression of the apoptotic DBD (Fig. 5A). Under these conditions and in the presence of 20 μM MMNG (a potent DNA damaging agent), we see that the expression of the apoptotic DBD results in the inhibition of endogenous PARP-1 activity to almost half of its normal levels (Fig. 5B). Similarly, we observe an inhibition of poly(ADP-ribose) synthesis of 30-40% in the presence of the apoptotic DBD when the dose of MNNG is increased to 40 and 60 μM.

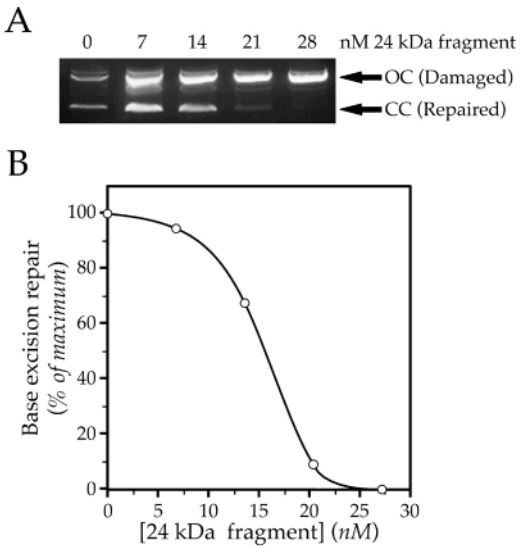

Fig. 4. Transdominant inhibition of DNA base excision repair by the N-terminal apoptotic fragment of PARP-1. DNA BER was assayed by monitoring the repair of oxy-radical damaged plasmids in whole cell extracts from the normal lymphoblastoid cell line GM01953C. (A) Agarose gel showing the repair of nicked plasmids (OC) following incubation in whole cell extract containing growing concentrations of the N-terminal apoptotic fragment of PARP-1. (B) Densitometric integration of the gel shown in (A). Repair is expressed as the proportion of repaired plasmids (CC) over the total amount of plasmids used in the repair reaction (OC+CC). Experiments were performed in the presence of a saturating concentration of NAD^+ (2 mM).

Interestingly, we observe a small but significant difference in the ability of the apoptotic and full-length DBDs of PARP-1 to inhibit poly(ADP-ribosyl)ation in vivo. Indeed, the levels of inhibition obtained with the apoptotic DBD are consistently superior (by ~15%) to the levels obtained with the full-length DBD of PARP-1 (data not shown; F.R.S., S. Kaufmann and G.G.P., unpublished). It is important to emphasize here that the levels of expression of both DBD are such that they do not completely inhibit poly(ADP-ribose) synthesis (Fig. 5B). This point is very important because it allows us to compare the activity of the DBDs under non-saturating conditions. This also explains the reduction in the inhibition of poly(ADP-ribose) synthesis seen at higher doses of MNNG and is consistent with the presence of increased level of DNA breaks relative to the DBDs.

DISCUSSION

In this study, we provide strong in vitro and in vivo evidence that caspase-mediated processing of PARP-1 effectively results in potent and irreversible inactivation of the DNA damage-dependent catalytic activity of PARP-1. We also demonstrate that this proteolytic inhibition is achieved by two distinct mechanisms, namely catalytic inactivation and dominant-negative inhibition of uncleaved PARP-1 and BER factors. We propose that this cleavage is a critical event during DNA damage-induced apoptosis as it prevents necrosis induced by poly(ADP-ribosyl)ation and survival induced by DNA repair.

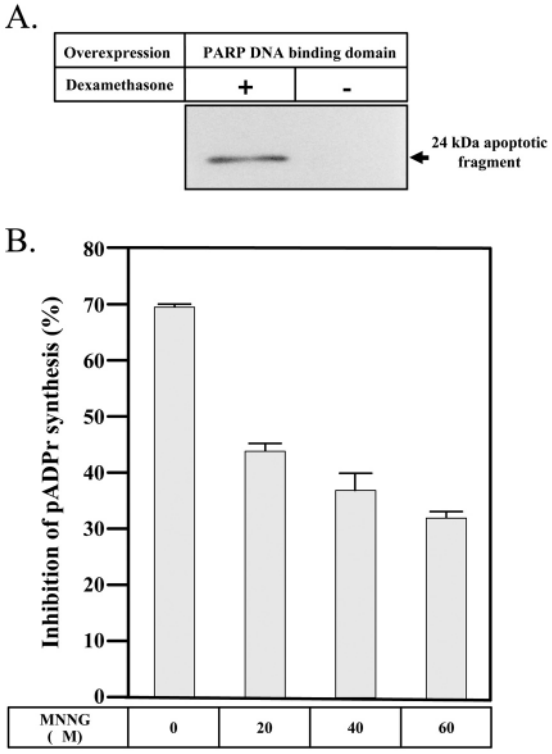

Fig. 5. Inhibition of poly(ADP-ribose) synthesis in cells by overexpression of the apoptotic (24 kDa) DBD of PARP-1. (A) Immunoblotting of the overexpressed DBD of PARP-1. Cultured cells were incubated for 16 hours with 1 mM dexamethasone to induce the expression of the DBD, and then extracted in SDS-PAGE loading buffer. 100,000 cells were loaded per well and analyzed by western blotting and immnodetection with the monoclonal antibody (F1-23), which recognizes the second zinc finger of PARP-1. (B) Transdominant inhibition of poly(ADP-ribose) synthesis by the apoptotic DBD of PARP-1 in cells treated with MNNG for 30 minutes. Different concentrations of MNNG were used as indicated on the histogram. The inhibition of pADPr synthesis is expressed as the % fraction of polymers detected in the presence of the 24 kDa DBD (+dexamethasone) over that detected in the absence of the fragment (–dexamethasone). The polymer levels detected in the absence of dexamethasone induction represent 100% PARP activity and are as follows: 185±3.7 units, 0 μM MMNG; 215±8.7 units, 20 μM MMNG; 430±17.3 units, 40 μM MMNG; and 837±15.6 units, 60 μM MMNG. Polymer levels were measured by quantitative immunodot blot (Affar et al., 1998) and are expressed as arbitrary fluorescence units per 4×10^6 cells. The results plus s.d. of three independent experiments are shown.

Interestingly, we did not observe any significant effect of the 89 kDa fragment of PARP-1 with any of the biochemical assays used in this study (data not shown). This might indicate that this fragment has only a minor or a more subtle role in the execution phase of apoptosis. This interpretation of our data is in agreement with the observation that the C-terminal fragment of PARP-1 is actively excluded from nuclear subdomains that are important for the initiation of the apoptotic process (Stegh et al., 1998; Alvarez-Gonzalez et al., 1999). Alternatively, this might also suggest that the 89 kDa apoptotic fragment needs to be in a cellular context to mediate its activity, possibly through interactions with other factors (Kim et al., 2000a).

At the energetic level, it is clear that the proteolytic inactivation of PARP-1 reported here will result in the prevention of NAD^+ depletion caused by PARP-1 activation. This observation is very important because NAD^+ depletion leads to ATP depletion, which is required for the execution phase of apoptosis (Berger, 1985; Szabo and Dawson, 1998). Indeed, when cellular ATP levels are artificially lowered, some uncharacterized aspect of the apoptotic program cannot be performed, thereby committing the dying cell to a necrotic demise (Eguchi et al., 1997; Leist et al., 1997). Previous studies have shown that poly(ADP-ribosyl)ation-dependent energy depletion can be achieved in no more than 20 minutes, but may also take up to several hours depending on the importance of the genomic insult sustained by cells (Berger, 1985; Oleinick and Evans, 1985). By contrast, cleavage of PARP-1 by caspases is initiated 30 to 60 minutes after the induction of apoptosis (Casiano et al., 1996, Kaufmann et al., 1993). It is clear from these kinetics that the cleavage of PARP-1 in cells suffering very high levels of DNA damage will be too late to prevent energy depletion and to antagonize necrosis, as already observed (Szabo and Dawson, 1998). However, in most cases where DNA damage is less intense, the cleavage of PARP-1 will be soon enough to preclude energy depletion and to ensure an apoptotic demise. The cleavage of PARP-1 and its subsequent inhibition might also play an important role in non-DNA damage-related apoptosis because the execution of the apoptotic program per se results in the nucleolytic degradation of genomic DNA (Wyllie, 1980). Although relatively late in the apoptotic process, the degradation of chromosomal DNA might result in massive PARP-1 activation, energy depletion and necrosis if not prevented by PARP-1 cleavage. This model is supported by the observation that poly(ADP-ribose) synthesis is stimulated in cells induced to undergo apoptosis by non-DNA damage-related stimuli (Simbulan-Rosenthal et al., 1998). Furthermore, this is in agreement with a recent study showing that expression of a caspase-resistant mutant of PARP-1 in cells treated with tumor necrosis factor α results in increased necrosis (Herceg and Wang, 1999).

Our results also show that DNA BER is strongly inhibited by the 24 kDa apoptotic fragment of PARP-1 (Fig. 4). The biological implications of this result are very important since this situation will artificially increase the actual number of DNA breaks in vivo in addition to those required initially to start the apoptotic program. The amplification of DNA damage seen under these conditions may act as a fail-safe mechanism to ensure that the damaged cells will not be able to repair their DNA and resume cell cycle to pass on potentially cancerogenous mutations. This mechanism is consistent with the fact that cell demise is not due to DNA damage per se at low doses but rather to the active ‘decision’ imposed on the cells by checkpoint proteins. Indeed, p53-deficient cells suffering DNA damage do not die of apoptosis as their p53-proficient counterpart, but repair the damage and continue to proliferate without obvious consequences (Griffiths et al., 1997). This indicates that the DNA repair proficiency of a cell can influence the decision to survive or die by apoptosis, particularly when a cell is at the threshold of inducing apoptosis, simply by decreasing the number of apoptosis-inducing lesions. This interpretation of our results is strengthened by the recent study of Halappanavar et al. (1999) who showed that cells expressing caspase-resistant PARP-1 survive significantly better than their wild-type counterpart in

response to low doses of DNA damage (Oliver et al., 1998, Fig. 3C). This is most likely due to the fact that uncleaved PARP-1 is allowed to carry on facilitating DNA repair in cells that are not irreversibly committed to apoptosis despite having progressed through the initial stages of the pathway. The elucidation of this process will require further experimental work.

Previous studies have shown that overexpression of PARP-1 DBD promotes apoptosis under various conditions (D'Amours et al., 1999). However, all these studies have used larger versions of the DBD (Schreiber et al., 1995; Gaken et al., 1996) or fusion variants of the apoptotic DBD (Kim et al., 2000b), which may not reflect accurately the activity of the native apoptotic DBD of PARP-1. We have indeed observed that the in vitro kinetics of inhibition of poly(ADP-ribosyl)ation seen with the apoptotic DBD does not match the kinetics that can be inferred from the use of the full-length DBD in vivo (Schreiber et al., 1995). The presence of helix-turn-helix motifs in the 42 kDa DBD of PARP-1, and not in the apoptotic 24 kDa DBD, might explain this observation. Unlike the zinc fingers of PARP-1, this DNA-binding motif has no particular affinity for DNA strand breaks (D'Amours et al., 1999), which may alter the mode of DNA binding of the 42 kDa DBD relative to the 24 kDa DBD. To validate our biochemical observations and to demonstrate conclusively the transdominant behaviour of the apoptotic DBD in vivo, we have established a cell line expressing the untagged apoptotic DBD of PARP-1. As shown in Fig. 5B, overexpression of the DBD leads to a strong inhibition of poly(ADP-ribose) polymer synthesis in vivo. The efficiency of PARP-1 inhibition by the apoptotic DBD is inversely correlated with the intensity of the DNA damage suffered by the cells (Fig. 5B). This observation indicates that the increased levels of DNA strand-breaks obtained at higher doses of DNA damage titrates out the inhibitory activity of the apoptotic DBD by providing additional activating sites to the endogenous PARP-1. This titration effect has been previously reported in systems using the full-length DBD and is consistent with a similar mode of action of both apoptotic and full-length DBDs at the mechanistic level. While this manuscript was in preparation, it was reported that overexpression of the apoptotic DBD of PARP stimulates apoptosis (Yung and Satoh, 2001), which is in agreement with our observation that this fragment is a potent inhibitor of poly(ADP-ribosyl)ation in vivo. In fact, we believe that the apoptotic DBD represents a good specific inhibitor of PARP-1 activity when expressed at high levels. However, it is worth mentioning that it is impossible to completely inhibit poly(ADP-ribose) synthesis in vivo by this approach because of the presence of additional PARP enzymes that can presumably be activated in different ways (Shall and de Murcia, 2000).

Taken together, the results presented in this report indicate that cleavage of PARP-1 by caspases is a highly significant event during the execution phase of the apoptotic program. Indeed, this proteolytic event results in a gain-of-function of the apoptotic fragment containing the DBD of the enzyme, which consequently causes a strong transdominant inhibition of poly(ADP-ribose) synthesis and a total abrogation of DNA BER. Our results support a model in which cleavage of PARP-1 promotes apoptosis by preventing DNA repair-induced survival and by blocking energy depletion-induced necrosis.

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