Apopain/CPP32 Cleaves Proteins That Are Essential for Cellular Repair: A Fundamental Principle of Apoptotic Death

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Summary

Proteolysis mediated by the interleukin 1β-converting enzyme (ICE) homologues is an important mechanism of the apoptotic process. The ICE homologue apopain/CPP-32/Yama (subsequently referred to as apopain) cleaves poly(ADP-ribose)polymerase (PARP) early during apoptosis. Additional apoptosis-specific protein cleavages have been observed in which the direct involvement of ICE-like proteases has been postulated. These substrates include the 70-kD protein component of the U1-ribonucleoprotein (U1-70kD), and the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs). The present studies demonstrate that U1-70kD and DNA-PKcs are excellent substrates for apopain, with cleavage occurring at sites that are highly similar to the cleavage site within PARP. The fragments generated from isolated protein substrates by apopain are identical to those observed in intact apoptotic cells, in apoptotic cell extracts, and in normal cell extracts to which apopain has been added. Like PARP, cleavage of these substrates in apoptotic cell extracts is abolished by nanomolar concentrations of Ac-DEVD-CHO and micromolar amounts of Ac-YVAD-CHO, confirming the involvement of apopain or an apopain-like activity. We propose that a central function of apopain or similar homologues in apoptosis is the cleavage of nuclear repair proteins, thereby abolishing their critical homeostatic functions.

Proteolytic cleavage of key substrates is an important biochemical mechanism underlying the apoptotic process, and the centrality of IL-1β-converting enzyme (ICE)-like proteases as mediators of apoptosis has been emphasized (1–3). In previous studies, we have shown that a subset of infrequently targeted autoantigens is united by their specific proteolytic cleavage early during apoptosis (4, 5). The kinetics and inhibition characteristics of these cleavages suggested the involvement of an ICE-like protease(s), but the specific substrate cutting sites and actual enzymatic activity(ies) responsible are unknown (5).

Apopain (CPP32) is an ICE homologue (6) that has recently been shown to cleave poly(ADP-ribose) polymerase (PARP) during apoptosis (7, 8). Specific inhibition of apopain (or apopain-like) activity attenuates apoptosis in vitro, strongly suggesting that proteolytic activity of this enzyme is mechanistically important in the apoptotic process (8). Apopain is highly selective for its macromolecular and peptide substrates (8), suggesting that it is responsible for the cleavage of other substrates during apoptosis, these substrates would share similar cleavage sites. We demonstrate here that apopain cleaves the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), the 70-kD protein component of the U1-ribonucleoprotein (U1-70kD), and PARP with comparable catalytic efficiencies. Furthermore, apopain cleaves all three substrates at very similar sites, defining a DXXD motif as the key determinant for cleavage specificity by apopain. Since U1-70kD (9, 10), PARP (11), and DNA-PKcs (12–14) function in the splicing of mRNA and the repair of double-strand DNA breaks, we suggest...
that a central role of apopain is the targeted abolition of these essential homeostatic pathways during apoptosis.

Materials and Methods

Cell Culture and Induction of Apoptosis. HeLa cells were passaged in 10% heat-inactivated calf serum using standard tissue culture procedures. Apoptosis was induced by irradiation with UVB as previously described (15).

In Vitro Cleavage of Purified DNA-PKcs and [35S]Methioninelabeled U1-70kD and PARP. cDNAs for U1-70kD (16) and PARP (17) were used to drive the synthesis of [35S]methionine-labeled proteins by coupled transcription/translation, and cleavage reactions were performed in the presence or absence of 42 pM apopain, as described previously for PARP (8). After incubation at 37°C for the indicated times, reactions were terminated, samples were electrophoresed on 12% SDS-polyacrylamide gels, and intact proteins and fragments were visualized by fluorography. In vitro cleavage of purified DNA-PKcs, was performed similarly using 30 nM DNA-PKcs (Promega, Madison, WI), 42 pM apopain, 2 mM MgCl2, and 10 μg/ml sheared herring sperm DNA (Promega) in the presence or absence of 150 μM ATP. Addition of DNA, Mg2+, and ATP to cleavage reactions containing apopain and PARP or U1-70kD had no effect on cleavage (data not shown), confirming that the cofactors acted on DNA-PKcs, rather than on the protease. Samples were electrophoresed on 10% SDS-polyacrylamide gels containing 0.087% bisacrylamide. Immunoblots were performed with serum AG (5) to detect the intact DNA-PKcs, and the 160-kD fragment. Identical results were obtained with mAb 18-2 (18), which recognizes the intact protein and the 250-kD fragment. All reactions were carried out using subsaturating levels of substrate (<Km), where the appearance of product is assumed to be a first-order process. The densities of substrate and product bands on autoradiograms were determined on a PDI Discovery densitometry system (Protein Databases, Inc., Huntington Station, NY) with Quantity One software (Protein Databases, Inc.). Time courses were fit to the first-order rate equation percent of substrate cleavage = 100-(1-e^{-k_{cat}[E]/K_{m}t}), to obtain a value for the catalytic constant kcat(Km).

In Vitro Cleavage of Endogenous U1-70kD, PARP, and DNA-PKcs. Control or early apoptotic HeLa cells (incubated for 3 h after UVB irradiation) were washed in Krebs Ringer buffer (KRB), and then harvested by scraping into KRB, followed by centrifugation at 400 g. The cell pellet was lysed on ice in 1 ml of lysis buffer containing 10 mM Hepes/KOH, pH 7.4, 2 mM EDTA, 5 mM DTT, 1% NP-40, and the protease inhibitors PMSF, antipain, leupeptin, and pepstatin A. Protein concentrations of cell lysates were 3-4 mg/ml, and were obtained from six confluent 10-cm dishes of HeLa cells. To investigate the in vitro cleavage of endogenous U1-70kD, PARP, and DNA-PKcs, 25-μl aliquots of control or apoptotic cell lysate were incubated at 37°C in the presence (control lysate) or absence (apoptotic lysate) of 105 pM added apopain. Samples were electrophoresed on 10% SDS-polyacrylamide gels containing 0.087% bisacrylamide. Cleavage of the intact proteins was assayed by immunoblotting with monospecific human sera recognizing U1-70kD and PARP (5), or a mAb raised against DNA-PKcs, that detects the intact DNA-PKcs, and a 250-kD fragment in apoptotic cells (18).

Kinase Assay. Reaction mixtures (30-μl final volume) contained 10 mM Hepes, pH 7.4, 2 mM MgCl2, 10 mM KCl, 2.7 mM DTT, 150 μM ATP, 10 μg/ml DNA-PKcs, and 100 ng SP1 (Promega) in the absence or presence of 0.75 U/μl of apopain, 100 nM Ac-DEVD-CHO, or 100 nM Ac-YVAD-CHO. All reactions were preincubated on ice for 15 min in the absence of ATP (to facilitate binding of the inhibitors, where added, to apopain), and then at 37°C for 10 min (to allow apopain-mediated cleavage of DNA-PKcs). Kinase reactions were then initiated by adding 150 μM ATP containing 1.5 μCi [32P]ATP (3,000 Ci/mmol) to each sample. After incubating at 37°C for 10 min, reactions were ended by adding SDS gel buffer and boiling. Samples were analyzed by electrophoresis on 8% SDS-polyacrylamide gels, followed by autoradiography. Phosphorylation of Sp1 was in the linear range of detection for the first 20 min of the assay, while autophosphorylation was maximal within 5 min (data not shown). Autophosphorylated DNA-PKcs provided a convenient internal control for the status (intact vs. cleaved) of the kinase. Cleaved status of the kinase was also confirmed independently by immunoblotting experiments (data not shown).

Determination of the Cleavage Site within U1-70kD. The full-length cDNA clone for U1-70kD, ligated into the EcoRI site of pGEM-3Zf(+), was used to generate [35S]U1-70kD by coupled in vitro transcription/translation (T7 polymerase, rabbit reticuloocyte lysate; Promega) in the presence of either [35S]cytochrome c or [35S]methionine. The radiolabeled [35S]U1-70kD polypeptides were purified by Superdex-75/FPLC (Pharmacia Fine Chemicals, Piscataway, NJ) gel-permeation chromatography. Carboxy-terminal fragments of the U1-70kD polypeptide, corresponding to potential apopain cleavage sites (EAGG324A325 and DGPD341G342), were generated by PCR-directed template modification followed by in vitro transcription/translation. A cDNA encoding the MetPro329-Glu337 fragment (which corresponds to cleavage at EAGG324A325) was amplified with the sense (forward) synthetic oligonucleotide 5'-GGA ATT CAT GCC CCC TGA TGA TGG GCC TCC AGG G-3' plus the antisense (reverse) oligonucleotide 5'-GGA ATT CTC TTC ACT CCG GCG CAG CCT CCA TC-3' was used to drive the synthesis of [35S]U1-70kD cDNA (3 ng/μl) as template (0.025 U/μl Pwo polymerase [Boehringer Mannheim Biochemicals, Indianapolis, IN], 25 cycles of 1 min at 94°C, 1 min at 60°C, 45 s at 72°C). A cDNA encoding the MetPro341-Glu347 fragment (which corresponds to cleavage at DGPD341G342) was amplified the same way, except that the sense (forward) oligonucleotide was 5'-GGA ATT CAT GCC CCC TGA TGA TGG GCC TCC AGG G-3' plus the antisense (reverse) oligonucleotide 5'-GGA ATT CTC TTC ACT CCG CCG CAG CCT CCA TC-3' was used to drive the synthesis of the corresponding [35S]U1-70kD cDNA as template (0.025 U/μl Pwo polymerase [Boehringer Mannheim Biochemicals, Indianapolis, IN], 25 cycles of 1 min at 94°C, 1 min at 60°C, 45 s at 72°C). The resulting PCR fragments were purified, transfected into EcoRIl, then ligated into the EcoRI site of pBluescript II SK(+). (Stratagene, La Jolla, CA). After sequence verification, T7-oriented clones were used to drive the synthesis of the corresponding [35S]U1-70kD cDNA as template (0.025 U/μl Pwo polymerase [Boehringer Mannheim Biochemicals, Indianapolis, IN], 25 cycles of 1 min at 94°C, 1 min at 60°C, 45 s at 72°C). The resulting PCR fragments were purified, transfected into EcoRIl, then ligated into the EcoRI site of pBluescript II SK(+). (Stratagene, La Jolla, CA). After sequence verification, T7-oriented clones were used to drive the synthesis of the corresponding [35S]U1-70kD cDNA as template (0.025 U/μl Pwo polymerase [Boehringer Mannheim Biochemicals, Indianapolis, IN], 25 cycles of 1 min at 94°C, 1 min at 60°C, 45 s at 72°C). The resulting PCR fragments were purified, transfected into EcoRIl, then ligated into the EcoRI site of pBluescript II SK(+). (Stratagene, La Jolla, CA). After sequence verification, T7-oriented clones were used to drive the synthesis of the corresponding [35S]U1-70kD cDNA as template (0.025 U/μl Pwo polymerase [Boehringer Mannheim Biochemicals, Indianapolis, IN], 25 cycles of 1 min at 94°C, 1 min at 60°C, 45 s at 72°C). The resulting PCR fragments were purified.
translation in reticulocyte lysates, and the resulting [35S]methionine-labeled protein was incubated with apopain (0.04 U/µl; 42 pM). Two fragments of 40-kD and 22-kD were simultaneously generated (Fig. 1 A) with cleavage kinetics ($k_{cat}/K_m = 2.3 \times 10^6$ M$^{-1}$s$^{-1}$) very similar to those for the apopain-mediated cleavage of [35S]methionine-labeled PARP ($k_{cat}/K_m = 5.0 \times 10^6$ M$^{-1}$s$^{-1}$) (Fig. 1, A and B). In the presence of DNA, Mg$^{2+}$ and ATP, purified DNA-PKcs was also efficiently cleaved by 42 pM apopain; the $k_{cat}/K_m$ of $7.7 \times 10^6$ M$^{-1}$s$^{-1}$ was comparable to that for apopain-mediated cleavage of PARP (Fig. 1, A and B). Two major fragments of 160- and 250-kD (each immunoblotted by a different antibody) were generated (see Figs. 1 A and 3); these comigrated with the fragments formed during apoptosis in intact cells (see reference 5 and Fig. 3). In the absence of Mg$^{2+}$ or ATP, the 160-kD fragment was further processed to 120 kD (Fig. 1 A, middle panel); the latter fragment, however, was never observed in lysates of intact apoptotic cells (5). When DNA was also omitted from the reaction, cleavage of DNA-PKcs was ~10-fold less efficient (data not shown). The increased efficiency of cleavage of DNA-PK in the presence of DNA fragments in vitro suggests that the active DNA-PK holoenzyme, bound at DNA ends and nicks (19–21), is the physiologic target for cleavage by apopain.

To address the effects of cleavage on DNA-PKcs function, kinase activity was measured by quantitating DNA-dependent phosphorylation of the transcription factor Sp1 (22). When the DNA-PK holoenzyme was incubated with Sp1 in the presence of cofactors and [$γ^{32}$P]ATP, both the
polypeptides (MetPro326-Glu 437 and MetPro343-Glu 437, re-
products were then used for in vitro transcription/transla-
ion DNA-PKcs at DEVD2712-N2713 (Fig. 2 B).

pain, and the NH2-terminal sequence of the 160-kD frag-
ment was determined by conventional Edman microsequenc-
ment was observed in apoptotic cells or generated by incuba-
tion with [3SS]methionine to generate the corresponding 35S
fragment failed to detect radioactivity in the first 20 cycles
adjacent to the full-length UI:70kD cleaved in vitro with
aged by asp in the P1 position for this family of
requirement for Asp in the P1 position for this family of
This suggested that the cleavage site resided in the COOH-
terminal third of the molecule, where, given the absolute
sequence obtained placed the apopain cleavage site
within U1-70kD by comigration with U1-70kD carboxy-terminal
fragments (lanes 9—11). [3SS]methionine-labeled
U1-70kD and DNA-PKcs were phosphorylated (Fig. 1 C,
upper panel). Cleavage of DNA-PKcs by apopain pro-
duced a 50% decrease in Sp1 phosphorylation (Fig. 1 C,
lanes 1 and 2). Incubation with 100 nM Ac-DEVD-CHO
(Ki apopain = 0.35 nM) abolished apopain-mediated cleavage of DNA-PKcs and prevented the decrease in kinase ac-
tivity (Fig. 1 C, lane 4 vs. lane 1). In contrast, cleavage of
DNA-PKcs still occurred in the presence of 100 nM Ac-
YVAD-CHO (Ki apopain = 10 μM) and was associated with a
50% decrease in kinase activity (Fig. 1 C, lanes 2 and 3).

Apopain-mediated Cleavages of U1-70kD and DNA-PKcs
Occur at DGPD341-C342 and DEVD2712-N2713, Respectively.
The excellent cleavage of PARP, U1-70kD, and DNA-
PKcs by apopain, as well as the restricted substrate specific-
ity of apopain, suggested that these three substrates share a
common or similar DEVD-like cleavage site. To elucidate
the cleavage site of U1-70kD, we labeled an in vitro trans-
lation reaction with [3S]cysteine, since only a single cyste-
ine exists in U1-70kD (at position 39). When this protein
was cleaved with apopain, only the 40-kD fragment was
detected, in contrast to the detection of both fragments
when labeling with [3S]methionine (Fig. 2 A, lanes 1—8).
This suggested that the cleavage site resided in the COOH-
terminal third of the molecule, where, given the absolute

U1-70kD, DNA-PKcs, or PARP. In addition to the proven cleavage site in DNA-PKcs,
(DEVD-N, solid arrowhead), three other potential sites (after Asp324,
Asp331, and Asp338) are indicated (open arrowheads) which might give rise
to the 120-kD fragment seen after in vitro cleavage with apopain in the
absence of Mg2+ or ATP.

In Vitro Cleavage Fragments Are Identical to Those Detected in Intact Apoptotic Cells. To directly compare the fragments
generated by apopain activity on purified substrates with those observed in apoptotic cells or generated by incuba-
tion of apoptotic cell extracts, we established an in vitro cell lystate system that supported the activity of ICE-like
proteases. Although little specific cleavage of endogenous

Figure 2. Apopain-mediated cleavage of U1-70kD occurs between Asp341 and Gly342. (A) Cleavage of [3S]cysteine- vs. [3S]methionine-labeled
U1-70kD (lanes 1—8) and identification of the apopain cleavage site
within U1-70kD by comigration with U1-70kD carboxy-terminal
fragments (lanes 9—11). [3S]cysteine- (lanes 1—4) and [3S]methionine-labeled
U1-70kD (lanes 5—8) were cleaved with the indicated amounts of
apopain for 60 min at 37°C. Lane 9, [3S]methionine-labeled MetPro326-
Glu347; lane 10, full-length [3S]methionine-labeled U1-70kD cleaved for
60 min at 37°C with 0.25 U/μl of apopain; lane 11, [3S]methionine-labeled
MetPro343-Glu347. All samples were electrophoresed on SDS-polyacryl-
ide gels and visualized by fluorography. The COOH-terminal fragments mi-
grate aberrantly slow on SDS-PAGE, consistent with previous observa-
tions (16). (B) Location of apopain cleavage sites in U1-70kD, DNA-PKcs,
and PARP. In addition to the proven cleavage site in DNA-PKcs,
(DEVD-N, solid arrowhead), three other potential sites (after Asp324,
Asp331, and Asp338) are indicated (open arrowheads) which might give rise
to the 120-kD fragment seen after in vitro cleavage with apopain in the
absence of Mg2+ or ATP.
In contrast, the potent ICE inhibitor Ac-YVAD-CHO (24) was 10,000-fold less efficient at inhibiting cleavage of these substrates in all three settings; IC₅₀ values ranged from 5–10 μM (data not shown). Taken together, these data confirm that the activity responsible for cleavage of these substrates in apoptotic cells is apopain or apopain-like, rather than ICE-like.

Discussion

These studies define DNA-PKcs and U1-70kD as two novel apopain substrates. Both are cleaved in vitro with very similar kinetics to PARP, the other known substrate for apopain. The cleavage fragments generated by apopain on purified components are identical to those observed in intact apoptotic cells. Lysates of control (lane 1) or apoptotic (lane 2) HeLa cells were electrophoresed adjacent to in vitro cleavage reactions performed on control (lanes 3–5) or early apoptotic (lanes 6 and 7) HeLa lysates. Cleavage was initiated by incubating lysates at 37°C in the presence (lane 5) or absence (lanes 3, 4, 6, and 7) of exogenous apopain for the indicated times. U1-70kD, PARP, and DNA-PKcs were detected by immunoblotting. 53 μg of protein was electrophoresed in each lane. Data from a representative experiment are shown; similar data were obtained in 10 other experiments.

demonstrated that apopain and ICE differ in their specificities, as well as in their promiscuity toward peptide and macromolecular substrates. For example, while apopain is highly specific for PARP and does not cleave pro–IL-1β, even at 5000-fold excesses of the enzyme (8), ICE is more promiscuous, showing only a 50-100 fold preference for pro–IL-1β over PARP (23). Similarly, while there is a >10,000 fold selectivity by apopain for the Ac–DEVD-AMC over the Ac–YVAD–AMC fluorogenic substrate, the relative preference of ICE for Ac–YVAD–AMC over Ac–DEVD–AMC is only approximately fivefold (Thornberry, N.A., unpublished data). U1–70kD, DNA–PKcs, and PARP were all cleaved by purified ICE in vitro, albeit at ∼30–50-fold higher enzyme concentration than is required for the processing of these substrates by apopain (data not shown). To identify the activity responsible for the physiologic cleavage of these substrates during apoptosis, we determined the tetrapeptide aldehyde inhibition profile for cleavage of endogenous substrates in apoptotic extracts (Fig. 4). Ac–DEVD–CHO was a potent inhibitor of cleavage of endogenous U1–70kD, DNA–PKcs, and PARP in apoptotic HeLa lysates (IC₅₀ = 0.2–1 nM; Fig. 4, lowest panel), and of apopain-mediated cleavages in control lysates (IC₅₀ = 0.2–0.5 nM; Fig. 4, middle panel). These effects of Ac–DEVD–CHO were identical to those observed for inhibition of apopain-mediated cleavage of the purified substrates (IC₅₀ = 0.1–0.4 nM; Fig. 4, upper panel). In contrast, the potent ICE inhibitor Ac–YVAD–CHO (24) was 10,000-fold less efficient at inhibiting cleavage of these substrates in all three settings; IC₅₀ values ranged from 5–10 μM (data not shown). Taken together, these data confirm that the activity responsible for cleavage of these substrates in apoptotic cells is apopain or apopain-like, rather than ICE-like.

Figure 3. In vitro cleavage fragments are identical to those observed in intact apoptotic cells. Lysates of control (lane 1) or apoptotic (lane 2) HeLa cells were electrophoresed adjacent to in vitro cleavage reactions performed on control (lanes 3–5) or early apoptotic (lanes 6 and 7) HeLa lysates. Cleavage was initiated by incubating lysates at 37°C in the presence (lane 5) or absence (lanes 3, 4, 6, and 7) of exogenous apopain for the indicated times. U1–70kD, PARP, and DNA–PKcs were detected by immunoblotting. 53 μg of protein was electrophoresed in each lane. Data from a representative experiment are shown; similar data were obtained in 10 other experiments.

Figure 4. Inhibition of apopain-mediated U1–70kD, DNA–PKcs, and PARP cleavages by Ac–DEVD–CHO. (Top panel) In vitro apopain-mediated cleavage of [³⁵S]methionine–labeled U1–70kD, [³⁵S]methionine–labeled PARP, or purified DNA–PKcs, is inhibited by Ac–DEVD–CHO; IC₅₀ = 0.1–0.4 nM. (Middle panel) Ac–DEVD–CHO inhibits the in vitro cleavage of endogenous substrates in control lysates (incubated in the presence of added purified apopain); IC₅₀ = 0.2–0.5 nM. (Lowest panel) Ac–DEVD–CHO inhibits the endogenous protease–mediated cleavages in early apoptotic lysates with IC₅₀ values of 0.2–1 nM. Squares, U1–70kD; triangles, PARP; circles, DNA–PKcs. The experiments were repeated on two to three separate occasions with identical results.
tact apoptotic cells or generated in lysates of apoptotic cells incubated in vitro. The tetrapeptide aldehyde inhibition profile of substrate cleavage in extracts of apoptotic cells showed IC_{50} = 0.2-1 nM for Ac-DEVD-CHO, but IC_{50} = 5-10 μM for Ac-YVAD-CHO, confirming that apopain or a very closely related activity is responsible for the physiological cleavage of these substrates in apoptotic cells.

All three apopain substrates share extremely similar cleavage sites, comprising DEVD-N in DNA-PKcs, DGPD-G in U1-70kD, and DEVD-G in PARP. In all cases, aspartate is found in P1 and P4, defining a DXXD motif as the key determinant for specificity of cleavage by apopain. These sites differ at the P3 position from those sites efficiently cleaved in proIL-1β by ICE (FEAD-G and YVHD-A) (25, 26). Strikingly, cleavage separates key functional domains of the molecule in all three cases (Fig. 2 B): (a) the RNA-binding domain from the distal arginine-rich region of U1-70kD (16); (b) the NH2-terminal domains containing a leucine zipper motif from the COOH-terminal PI3 kinase–like domain of DNA-PKcs (27); and (c) the DNA-binding domain from the catalytic and automodification domains of PARP (28).

Cells are equipped with several complex strategies to repair various types of DNA damage. These include (a) the sensing of DNA damage by a family of PI3 kinase–like proteins (29), and potentially by PARP (11); and (b) the activation of several checkpoint proteins (e.g., p53), that arrest the cell cycle and thereby allow DNA repair to occur by base excision repair pathways (30), nucleotide excision repair pathways (31), mismatch repair pathways (32), and pathways for double-strand DNA break repair (27). It is of great interest that DNA-PKcs and PARP are substrates for apopain, and that several other proteins also possess DXXD sequences and might conceivably be substrates for apopain during apoptosis. These include (a) uracil DNA glycosylase (involved in base-excision repair and contains a DIED-F sequence at an exposed loop at positions 180–184 [33]); (b) the PI3 kinase homologue FBKP rapamycin-associated protein (FRAP) (implied in mediating G1 cell cycline progression (34), and contains a DHTD-G sequence at residues 1538–1542 and a DLLD-A sequence at residues 1569–1573); and (c) the PI3 kinase homologue ATM (mutated in ataxia telangiectasia and postulated to function in cell cycle control, and contains a DIVD-G sequence at residues 1565–1569 of the partial cDNA sequence [35]). It will be important to address whether these proteins are cleaved by apopain in apoptotic cells. Since the cellular machinery necessary to sense and repair DNA damage would counteract the DNA degradation characteristic of apoptosis, destruction of this machinery would be physiologically essential if the decision to die is to be irreversible and rapidly executed.

Mutations in several of the proteins involved in DNA repair discussed above (e.g., DNA-PKcs, ATM) have been associated with severe phenotypes and/or susceptibility to malignancy, suggesting that the abolition of their function severely impairs DNA repair processes (27, 35, 36). In contrast, the phenotype observed in PARP knockout mice was minimal (37), suggesting that PARP function in DNA repair is redundant, and hence that its proteolysis in apoptosis does not exert any functional effect by loss of function alone. The possibility that one of the apoptotic PARP fragments has a dominant negative role in abrogating DNA repair has been suggested (38), but remains to be directly addressed.

Overexpression of the COOH-terminal domain of U1-70kD (which contains two arginine/serine-rich [SR] regions) has a dominant negative effect on splicing and transport of mRNA to the cytoplasm (39). Interestingly, the 22-kD fragment generated by apopain contains one of these SR domains, and would likely have a similar effect. Since repair pathways depend on new mRNA synthesis, inhibition of mRNA splicing during apoptosis might impair expression of the homeostatic transcriptional response. Domains similar to the SR domain in U1-70kD are also found in an extended family of splicing factors, the SR proteins (40). It is therefore of great interest that five members of this SR family contain a DXXD sequence just upstream of their SR domains (40). It will be important to address whether these proteins are also cleaved during apoptosis, potentially liberating many SR domains and thereby abolishing mRNA splicing. The cleavage of nuclear lamin by an ICE-like enzyme during the later phases of apoptosis has recently been recognized (41, 42), and might facilitate nuclear condensation and fragmentation during the later phases of this process. Since these intermediate filament proteins have a role in nuclear envelope integrity and the organization of interphase chromatin (43), the altered nuclear structure might also limit those functions (e.g., transcription and mRNA splicing) that are topographically organized.

The specific proteolytic cleavages and DNA breaks that proceed rapidly during the execution phase of apoptosis imply that macromolecule degradation far outpaces repair (3). We propose that the efficient cleavage by a single, highly specific protease of three nuclear proteins involved in homeostatic pathways eliminates essential repair functions. The focused crippling of homeostasis by apopain (and potentially other similar homologues) may be a fundamental feature ensuring the rapid irreversibility of the apoptotic process.

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