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-PK_{cs}). The present studies demonstrate that U1-70kD and DNA-PK_{cs} 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)[1]-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 if 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-PK_{cs}), 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-PK_{cs} (12–14) function in the splicing of mRNA and the repair of double-strand DNA breaks, we suggest

[1] *Abbreviations used in this paper:* Ac-YVAD-CHO, *N*-(*N*-Ac-Tyr-Val-Ala)-3-amino-4-oxobutanoic acid; Ac-DEVD-CHO, *N*-(N-Ac-Asp-Glu-Val)-3 amino-4-oxobutanoic acid; DNA-PK_{cs}, catalytic subunit of the DNA-dependent protein kinase; ICE, IL-1β–converting enzyme; KRB, Krebs Ringers buffer; PARP, poly (ADP-ribose) polymerase; SR, serine rich; U1-70kD, 70kD protein component of the U1 small nuclear ribonucleoprotein.

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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-PK$_{cs}$ and [^{35}S]Methionine-labeled U1-70kD and PARP. cDNAs for U1-70kD (16) and PARP (17) were used to drive the synthesis of [^{35}S]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-PK$_{cs}$ was performed similarly using 30 nM DNA-PK (Promega, Madison, WI), 42 pM apopain, 2 mM $MgCl_2$, and 10 μg/ml sheared herring sperm DNA (Promega) in the presence or absence of 150 μM ATP. Addition of DNA, Mg^{2+}, 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-PK$_{cs}$ 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-PK$_{cs}$ 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 ($<K_m$), 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 = $100*(1-e^{-(k_{cat}*[E]/K_m)*time})$, to obtain a value for the catalytic constant k_{cat}/K_m.

In Vitro Cleavage of Endogenous U1-70kD, PARP, and DNA-PK$_{cs}$. Control or early apoptotic HeLa cells (incubated for 3 h after UVB irradiation) were washed in Krebs Ringers 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-PK$_{cs}$, 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-PK$_{cs}$ that detects the intact DNA-PK$_{cs}$ 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 $MgCl_2$, 10 mM KCl, 2.7 mM DTT, 150 μM ATP, 10 μg/ml DNA, 50 ng DNA-PK, 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-PK$_{cs}$). Kinase reactions were then initiated by adding 150 μM ATP containing 1.5 μCi [^{32}P]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-PK$_{cs}$ 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 [^{35}S]U1-70kD by coupled in vitro transcription/translation (T7 polymerase, rabbit reticulocyte lysate; Promega) in the presence of either [^{35}S]cysteine or [^{35}S]methionine. The radiolabeled [^{35}S]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 (EAGD^{324}A^{325} and DGPD^{341}G^{342}), were generated by PCR-directed template modification followed by in vitro transcription/translation. A cDNA encoding the MetPro^{326}-Glu^{437} fragment (which corresponds to cleavage at EAGD^{324}A^{325}) 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′ using the full-length 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 MetPro^{343}-Glu^{437} fragment (which corresponds to cleavage at DGPD^{341}G^{342}) was amplified the same way, except that the sense (forward) oligonucleotide was 5′-GGA ATT CAT GCC AGA GGA AAA GGG CCG GGA TCG TG-3′ The resulting PCR fragments were purified, trimmed with EcoRI, 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 [^{35}S]U1-70kD COOH-terminal fragments by coupled in vitro transcription/translation, as described above.

Protease Inhibitor Experiments. Increasing concentrations of the tetrapeptide aldehyde inhibitor Ac-DEVD-CHO (8) were preincubated for 20 min on ice with apopain and/or HeLa cell lysates before allowing cleavage reactions to proceed at 37°C. Apopain-mediated cleavages of purified components were performed, visualized, and quantitated as described above. Cleavage of endogenous substrates was assayed in aliquots of HeLa cell lysate incubated in the presence (control lysate) or absence (apoptotic lysate) of 105 pM purified apopain. Maximum cleavage of the intact substrates, measured in the absence of Ac-DEVD-CHO, never exceeded 50%.

Results

Purified Apopain Cleaves U1-70kD and DNA-PK$_{cs}$ To directly address whether apopain cleaves U1-70kD, a cDNA for U1-70kD was used to direct in vitro transcription/

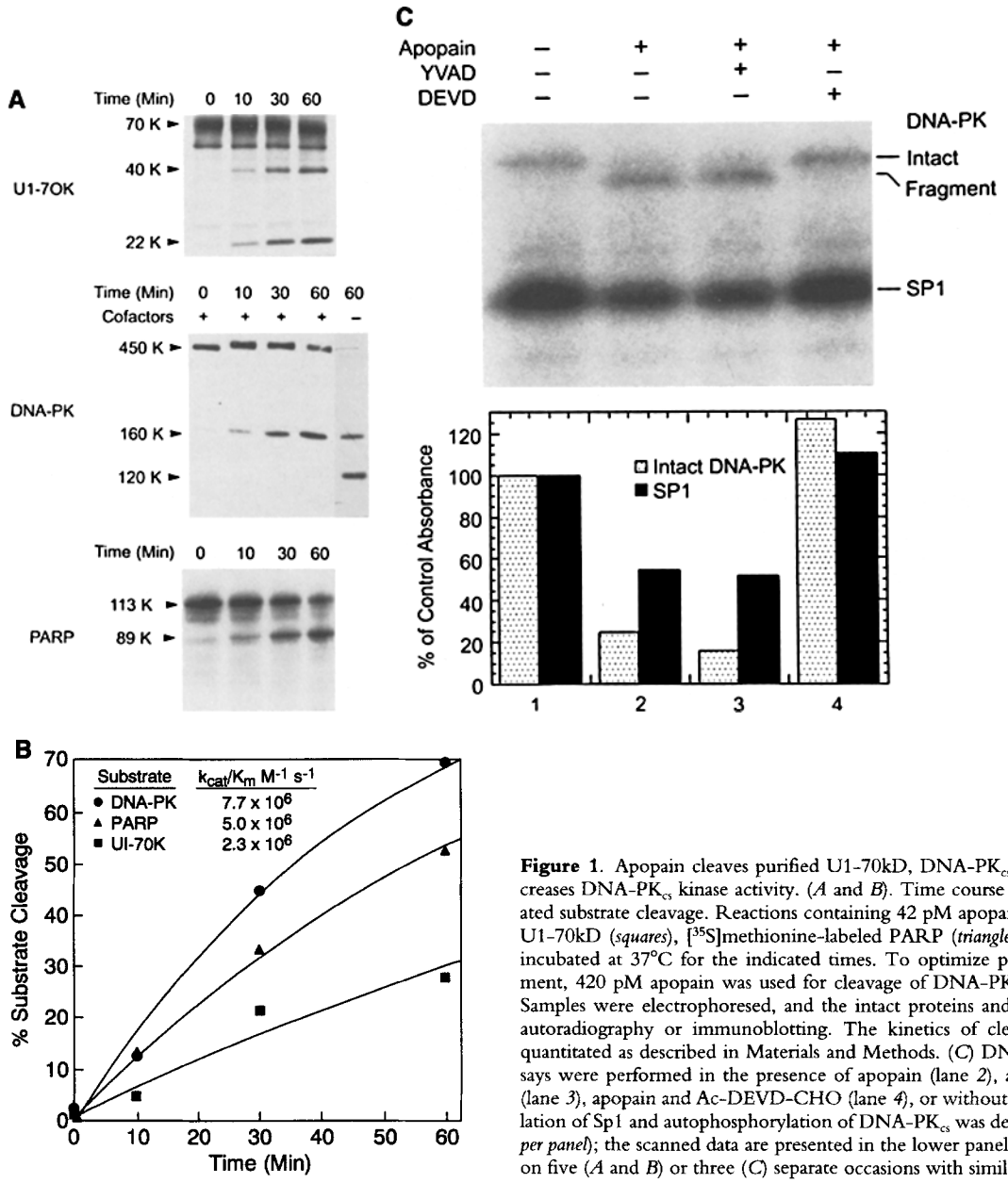

Figure 1. Apopain cleaves purified U1-70kD, DNA-PK$_{cs}$, and PARP in vitro, and decreases DNA-PK$_{cs}$ kinase activity. (*A* and *B*). Time course and kinetics of apopain-mediated substrate cleavage. Reactions containing 42 pM apopain and [^{35}S]methionine-labeled U1-70kD (*squares*), [^{35}S]methionine-labeled PARP (*triangles*), or DNA-PK$_{cs}$ (*circles*) were incubated at 37°C for the indicated times. To optimize processing to the 120-kD fragment, 420 pM apopain was used for cleavage of DNA-PK$_{cs}$ in the absence of cofactors. Samples were electrophoresed, and the intact proteins and fragments were visualized by autoradiography or immunoblotting. The kinetics of cleavage of these substrates was quantitated as described in Materials and Methods. (*C*) DNA-PK kinase assay. Kinase assays were performed in the presence of apopain (lane *2*), apopain and Ac-YVAD-CHO (lane *3*), apopain and Ac-DEVD-CHO (lane *4*), or without additions (lane *1*). Phosphorylation of Sp1 and autophosphorylation of DNA-PK$_{cs}$ was detected by autoradiography (*upper panel*); the scanned data are presented in the lower panel. Experiments were performed on five (*A* and *B*) or three (*C*) separate occasions with similar results.

translation in reticulocyte lysates, and the resulting [^{35}S]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 × 10^6 M^{-1}·s^{-1}) very similar to those for the apopain-mediated cleavage of [^{35}S]methionine-labeled PARP (k_{cat}/K_m = 5.0 × 10^6 M^{-1}·s^{-1}) (Fig. 1, *A* and *B*). In the presence of DNA, Mg^{2+} and ATP, purified DNA-PK$_{cs}$ was also efficiently cleaved by 42 pM apopain; the k_{cat}/K_m of 7.7 × 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-PK$_{cs}$ 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-PK$_{cs}$ 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

substrate and DNA-PK_{cs} itself were phosphorylated (Fig. 1 *C, upper panel*). Cleavage of DNA-PK_{cs} by apopain produced a 50% decrease in Sp1 phosphorylation (Fig. 1 *C*, lanes *1* and *2*). Incubation with 100 nM Ac-DEVD-CHO (K_i apopain = 0.35 nM) abolished apopain-mediated cleavage of DNA-PK_{cs} and prevented the decrease in kinase activity (Fig. 1 *C*, lane *4* vs. lane *1*). In contrast, cleavage of DNA-PK_{cs} still occurred in the presence of 100 nM Ac-YVAD-CHO (K_i 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-PK_{cs} Occur at $DGPD^{341}$-G^{342} and $DEVD^{2712}$-N^{2713}, Respectively. The excellent cleavage of PARP, U1-70kD, and DNA-PK_{cs} by apopain, as well as the restricted substrate specificity 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 translation reaction with [^{35}S]cysteine, since only a single cysteine 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 [^{35}S]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 requirement for Asp in the P_1 position for this family of proteases, there are two potential sites that could give rise to appropriately sized fragments (after Asp^{324} or Asp^{341}). NH_2-terminal radiosequencing of the ^{3}H-leucine-labeled 22-kD fragment failed to detect radioactivity in the first 20 cycles of Edman degradation, suggesting that apopain-mediated cleavage occurred at the Asp^{341} site (since the Asp^{324} cleavage would generate a fragment with leucine in position 11). Owing to a lack of suitable radiosequencing amino acids downstream of this site, appropriate truncations of the U1-70kD cDNA were generated, which encoded the COOH-terminal portions of U1-70kD predicted to arise from cleavage between Asp^{324}-Ala^{325} or Asp^{341}-Gly^{342}. These PCR products were then used for in vitro transcription/translation with [^{35}S]methionine to generate the corresponding ^{35}S polypeptides ($MetPro^{326}$-Glu^{437} and $MetPro^{343}$-Glu^{437}, respectively). The truncated products were electrophoresed adjacent to the full-length U1-70kD cleaved in vitro with apopain. The smaller product (corresponding to cleavage after Asp^{341}) comigrated with the 22-kD fragment generated by apopain, placing the cleavage at $DGPD^{341}$-G^{342} (Fig. 2 *A*, lanes *9–11*). To determine the cleavage site of DNA-PK_{cs}, purified kinase was cleaved in vitro with apopain, and the NH_2-terminal sequence of the 160-kD fragment was determined by conventional Edman microsequencing. The sequence obtained placed the apopain cleavage site in DNA-PK_{cs} at $DEVD^{2712}$-N^{2713} (Fig. 2 *B*).

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 incubation of apoptotic cell extracts, we established an in vitro cell lysate system that supported the activity of ICE-like proteases. Although little specific cleavage of endogenous

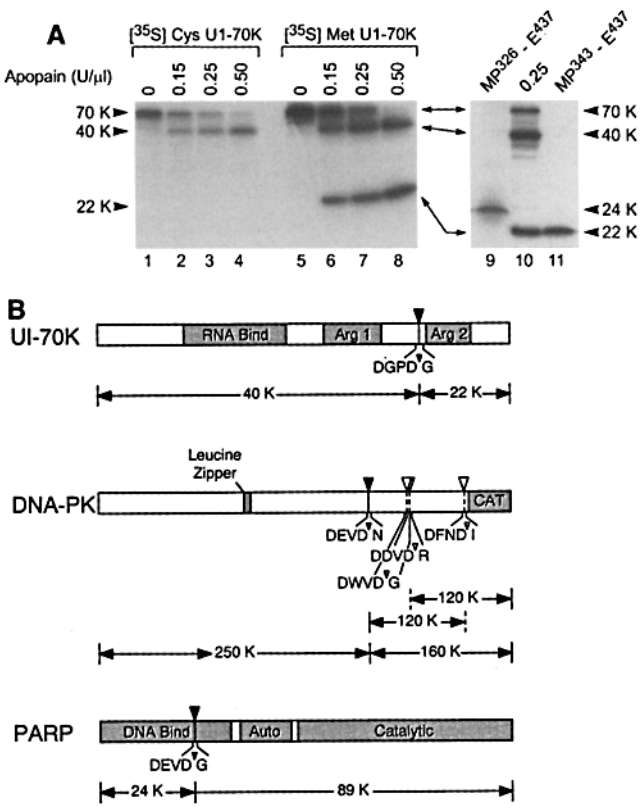

Figure 2. Apopain-mediated cleavage of U1-70kD occurs between Asp^{341} and Gly^{342}. (*A*) Cleavage of [^{35}S]cysteine- vs. [^{35}S]methionine-labeled U1-70kD (lanes *1–8*) and identification of the apopain cleavage site within U1-70kD by comigration with U1-70kD carboxy-terminal fragments (lanes *9–11*). [^{35}S]cysteine- (lanes *1–4*) and [^{35}S]methionine-labeled U1-70kD (lanes *5–8*) were cleaved with the indicated amounts of apopain for 60 min at 37°C. Lane *9*, [^{35}S]methionine-labeled $MetPro^{326}$-Glu^{437}; lane *10*, full-length [^{35}S]methionine-labled U1-70kD cleaved for 60 min at 37°C with 0.25 U/μl of apopain; lane *11*, [^{35}S]methionine-labeled $MetPro^{343}$-Glu^{437}. All samples were electrophoresed on SDS-polyacrylamide gels and visualized by fluorography. The COOH-terminal fragments migrate aberrantly slowly on SDS-PAGE, consistent with previous observations (16). (*B*) Location of apopain cleavage sites in U1-70kD, DNA-PK_{cs}, and PARP. In addition to the proven cleavage site in DNA-PK_{cs} (DEVD-N, *solid arrowhead*), three other potential sites (after Asp^{2982}, Asp^{3096}, and Asp^{3660}) are indicated (*open arrowheads*) which might give rise to the 120-kD fragment seen after in vitro cleavage with apopain in the absence of Mg^{2+} or ATP.

U1-70kD, DNA-PK_{cs}, or PARP was detected upon incubation of control (nonapoptotic) HeLa cell lysates in vitro (Fig. 3, lanes *3* and *4*), significant cleavage of these substrates occured when lysates made from early apoptotic HeLa cells were incubated at 37°C (Fig. 3, lanes *6* and *7*). Efficient cleavage of all endogenous substrates could also be induced in control lysates by addition of 105 pM apopain (Fig. 3, lane *5*). All fragments generated in control or apoptotic cell lysates using exogenous or endogenous apopain, respectively, comigrated exactly with those observed in intact apoptotic cells (Fig. 3, compare lanes *2*, *5*, and *7*). Identical findings were made using lysates of human keratinocytes or an osteosarcoma cell line (data not shown).

Ac-DEVD-CHO Inhibits the Apopain-mediated Cleavage of U1-70kD, DNA-PK_{cs}, and PARP. Previous studies have

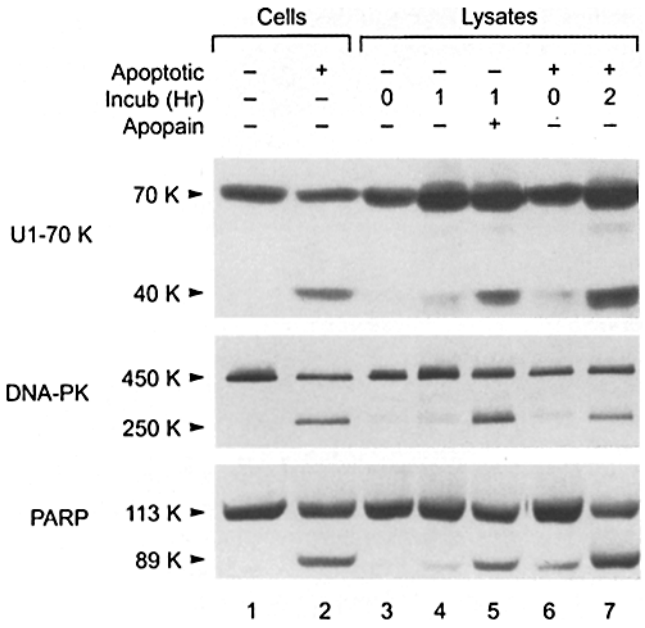

Figure 3. In vitro cleavage fragments are identical to those observed in intact apoptotic cells. Lysates of control (lane *1*) or apoptotic (lane *2*) HeLa cells were electrophoresed adjacent to *in vitro* cleavage reactions performed on control (lanes *3–5*) or early apoptotic (lanes *6* and *7*) HeLa 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-PK_{cs} 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-PK_{cs}, 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-PK_{cs}, and PARP in apoptotic HeLa lysates (IC_{50} = 0.2–1 nM; Fig. 4, *lowest panel*), and of apopain-mediated cleavages in control lysates (IC_{50} = 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 (IC_{50} = 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; IC_{50} 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-PK_{cs} 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 in-

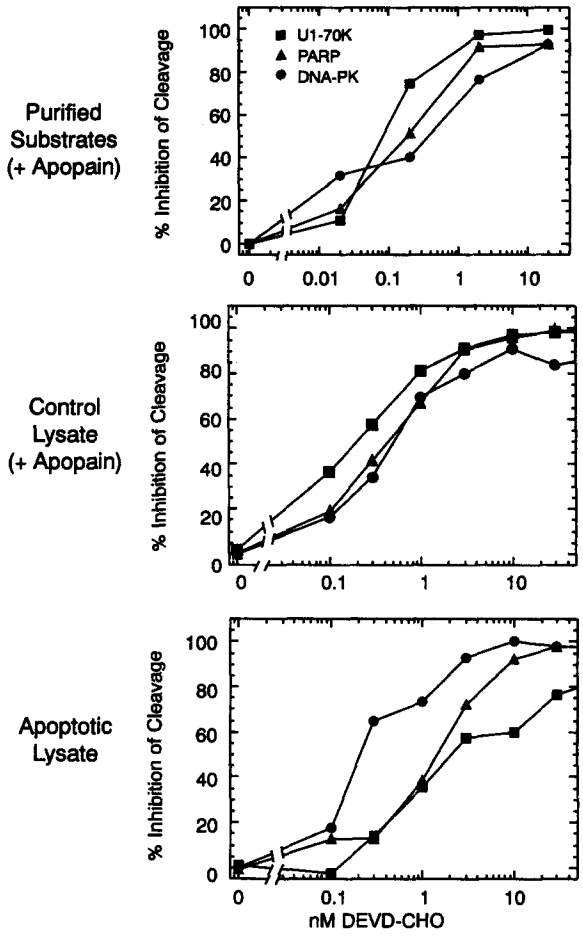

Figure 4. Inhibition of apopain-mediated U1-70kD, DNA-PK_{cs}, and PARP cleavages by Ac-DEVD-CHO. (*Top panel*) In vitro apopain-mediated cleavage of [^{35}S]methionine-labeled U1-70kD, [^{35}S]methionine-labeled PARP, or purified DNA-PK_{cs} is inhibited by Ac-DEVD-CHO; IC_{50} = 0.1–0.4 nM. (*Middle panel*) Ac-DEVD-CHO inhibits the in vitro cleavage of endogenous substrates in control lysates (incubated in the presence of added purified apopain); IC_{50} = 0.2–0.5 nM. (*Lowest panel*) Ac-DEVD-CHO inhibits the endogenous protease-mediated cleavages in early apoptotic lysates with IC_{50} values of 0.2–1 nM. *Squares*, U1-70kD; *triangles*, PARP; *circles*, DNA-PK_{cs}. 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 physiologic cleavage of these substrates in apoptotic cells.

All three apopain substrates share extremely similar cleavage sites, comprising DEVD-N in DNA-PK_{cs}, DGPD-G in U1-70kD, and DEVD-G in PARP. In all cases, aspartate is found in P_1 and P_4, defining a DXXD motif as the key determinant for specificity of cleavage by apopain. These sites differ at the P_4 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 NH_2-terminal domains containing a leucine zipper motif from the COOH-terminal PI3 kinase–like domain of DNA-PK_{cs} (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-PK_{cs} 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 FKBP rapamycin-associated protein (FRAP) (implicated in mediating G1 cell cycle 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-PK_{cs}, 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 lamins 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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