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Heteronuclear Ribonucleoproteins C1 and C2, Components of the Spliceosome, Are Specific Targets of Interleukin 1β-converting Enzyme-like Proteases in Apoptosis*

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Apoptosis induced by a variety of agents results in the proteolytic cleavage of a number of cellular substrates by enzymes related to interleukin 1β-converting enzyme (ICE). A small number of substrates for these enzymes have been identified to date, including enzymes involved in DNA repair processes: poly(ADP-ribose) polymerase and DNA-dependent protein kinase. We describe here for the first time the specific cleavage of the heteronuclear ribonucleoproteins (hnRNPs) C1 and C2 in apoptotic cells induced to undergo apoptosis by a variety of stimuli, including ionizing radiation, etoposide, and ceramide. No cleavage was observed in cells that are resistant to apoptosis induced by ionizing radiation. Protease inhibitor data implicate the involvement of an ICE-like protease in the cleavage of hnRNP C. Using recombinant ICE-like proteases and purified hnRNP C proteins *in vitro*, we show that the C proteins are cleaved by Mch3α and CPP32 and, to a lesser extent, by Mch2α, but not by ICE, Nedd2, Tx, or the cytotoxic T-cell protease granzyme B. The results described here demonstrate that the hnRNP C proteins, abundant nuclear proteins thought to be involved in RNA splicing, belong to a critical set of protein substrates that are cleaved by ICE-like proteases during apoptosis.

Apoptosis or programmed cell death plays an essential role in development, homeostasis, and defense in multicellular organisms. Inappropriate apoptosis may contribute to the pathology of many human diseases, including cancer. Despite its importance, however, much remains to be learned about the molecular events controlling this process. Several recent reviews describe advances in the field (1–6). The process of apoptosis can be initiated by a variety of stimuli and results in defined morphological changes such as nuclear condensation and membrane blebbing (7). Genetic analysis of the apoptotic pathway in the nematode *Caenorhabditis elegans* defined several genes that play essential roles in the execution of apoptosis (8, 9). The protein encoded by one of these genes, *ced*-3, is homologous to the interleukin 1β-converting enzyme (ICE)[1] family of cysteine proteases. A large number of ICE-like proteases have now been described (10–12) and when overexpressed in cells have been shown to induce apoptosis (13–16). Recent evidence suggests that at least one member of this family, FLICE/MACH/Mch5, binds directly with FADD, a protein that interacts with the activated Fas receptor, thus providing the first biochemical evidence physically linking a death receptor to an ICE-like protease (17–19). It is likely that once activated, FLICE acts on other ICE family members, which cleave cellular proteins. The individual roles of the ICE family of proteases in apoptosis remain to be elucidated.

A number of proteins have been shown to be degraded during apoptosis (20). These include the DNA repair enzymes poly(ADP-ribose) polymerase and DNA-dependent protein kinase (DNA-PK), both of which are cleaved by the ICE homolog CPP32 (21–26). Poly(ADP-ribose) polymerase can also be cleaved by other members of this protease family, including ICE, Tx, Nedd2, Mch-2α, Mch-3α, and FLICE (17, 23, 27–29). While poly(ADP-ribose) polymerase cleavage has been seen as the hallmark of apoptosis, it is not essential for the process, since poly(ADP-ribose) polymerase knockout mice develop normally (30). Other recently described death substrates that are cleaved by ICE-like enzymes include the U1 70-kDa protein component of the small nuclear ribonucleoprotein U1 snRNP (31, 32), protein kinase Cδ (33), sterol regulatory element-binding proteins (34, 35), lamin (36, 37) and Gas2 (38). In this study we provide the first evidence of cleavage of the hetero-

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[1] The abbreviation used are: ICE, interleukin 1β-converting enzyme; PK, protein kinase; hnRNP, heteronuclear ribonucleoprotein; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; YVAD-CMK, Tyr-Val-Ala-Asp-chloromethylketone; DEVD-CHO, Ac-Asp-Glu-Val aspartic acid aldehyde; PBS, phosphate-buffered saline; VAD-FMK, *Z*-Val-Ala-Asp-CH_2F; PIPES, 1,4-piperazinediethanesulfonic acid; HPLC, high performance liquid chromatography; CPP32, cysteine protease protein of molecular mass 32 kDa; Mch, mammalian Ced-3 homolog; MACH, MORT 1-associated CED-3/ice homolog; FADD, Fas-associated death domain protein; FLICE, FADD-like ICE; sn, small nuclear.

nuclear ribonucleoproteins (hnRNPs) C1 and C2 by ICE-like enzymes in apoptotic cells. The hnRNP C proteins are highly conserved among vertebrates, indicating an essential cellular function (39).

EXPERIMENTAL PROCEDURES

Reagents—Aprotinin, leupeptin, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), CHAPS, 4-(2-aminoethyl)benzene sulfonyl fluoride, proteinase K, and DNase-free RNase were obtained from Boehringer Mannheim. Phenylmethylsulfonyl fluoride (PMSF), iodoacetamide, *N*-ethylmaleimide, etoposide, β-glycerophosphate, benzamidine, Nonidet P-40, and EGTA were from Sigma. Tyr-Val-Ala-Asp-chloromethylketone (YVAD-CMK) and Ac-Asp-Glu-Val aspartic acid aldehyde (DEVD-CHO) were from Bachem. *Z*-Val-Ala-Asp-CH_2F (VAD-FMK) and *Z*-Asp-Glu-Val-Asp-(7-amino-4-trifluoromethyl coumarin) were from Enzyme Systems Products (Dublin, CA). Reduced Triton X-100 and C8 ceramide were from Calbiochem. Sequencing grade endoproteinase Lys-C was purchased from Promega. Tetrandrine, an anti-inflammatory, immunosuppressive compound extracted from the root of the creeper *Stephania tetranchra* and capable of causing apoptosis in lymphoid cells (40), was obtained from Professor Y. H. Thong (Department of Child Health, University of Queensland). Monoclonal antibodies to hnRNP C (4F4) and A1 (9H10) have been previously described (39, 41). Purified granzyme B was kindly provided by Dr. J. Trapani (Austin Research Institute, Melbourne, Australia). Electrophoresis materials were from Bio-Rad, and all other reagents were analytical grade.

Cell Culture—The cell lines referred to here, BL30A (susceptible to apoptosis) and BL30K (resistant to apoptosis), are an isogenic pair of Burkitt's lymphoma cell lines previously referred to as BL30 and BL30(s), respectively (42). BM13674 and BL29 are also Burkitt's lymphoma lines, with the latter being resistant to radiation-induced apoptosis (42). U937 is a monocytic cell line, and Molt-4 is a lymphocytic leukemia line. Cells were maintained at 37 °C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum in a 5% CO_2 atmosphere. BL30A cells were maintained under similar conditions using 20% fetal calf serum. For the induction of apoptosis cells were irradiated with 20 Gy using a ^{137}Cs radiation source or exposed to 68 μM etoposide, 50 μM C8 ceramide, 20 μg/ml tetrandrine, or 5 mM EGTA.

Detection of Apoptosis—2.5×10^5 cells were centrifuged (300 × *g* for 5 min) onto sterile 12-mm coverslips in 24-well plates. Sedimented cells were fixed in Carnoys fixative (6:1 ethanol:glacial acetic acid) for 5 min, recentrifuged, and fixed again for 5 min. The fixative was removed, and the plates were dried overnight at 37 °C. The cells were stained with 0.1 μg/ml Hoechst 33258 in citrate phosphate buffer (0.1 M citric acid and 0.2 M Na_2HPO_4, pH 5.5) for 5 min in the dark, washed, and then mounted. Cells were visualized using a Zeiss Axioskop fluorescent microscope, and 500 cells from each replicate were counted to determine the percentage of apoptosis (cells undergoing micronuclear fragmentation and condensation).

Apoptosis was also assessed by DNA fragmentation as visualized after extraction and electrophoresis of DNA. 5×10^5 cells were microfuged for 3 min, and the pellet was lysed in 25 μl of lysis buffer (0.5% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0, and 50 μg/ml proteinase K) and incubated at 50 °C for 1 h. 10 μl of DNase-free RNase A was added, and incubation continued for a further 1 h at 50 °C. Samples were heated to 70 °C in loading buffer (10 mM EDTA, 0.25% bromophenol blue, 40% sucrose, and 1% agarose (low melting point)) and were resolved on a 2% agarose Tris/borate/EDTA.

Preparation of Total Cellular, Cytoplasmic, and Nuclear Extracts—Total cell lysate was obtained by pelleting and washing the cells with cold phosphate-buffered saline (PBS). The cell pellet was lysed by rocking at 4 °C for 10 min in universal immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.2% Triton X-100, 0.3% Nonidet P-40, 0.1 mM sodium vanadate, 0.1 mM 4-(2-aminoethyl)benzene sulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM pepstatin, and 1 mM benzamidine). The solution was microfuged at 10,500 × *g* for 10 min, and the supernatant was stored at −70 °C.

Cytoplasmic extracts from BL30A cells were prepared essentially as described by Martin *et al.* (43). Briefly, the cells were incubated for 6 h after induction of apoptosis by 20-Gy irradiation and were then pelleted at 200 g. After washing twice in PBS, the cells were washed with 5 ml of cell extract buffer (50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 10 μM cytochalasin B, and 1 mM PMSF) and pelleted, and the supernatant was removed. An equal volume of cell extract buffer was added, and incubation continued on ice for 20 min. The cells were lysed gently with 20 strokes in a Dounce homogenizer, and the lysate was transferred to a 1-ml microcentrifuge tube and centrifuged at 4 °C for 15 min at 14,000 × *g*. The clear cytosol was carefully removed without disturbing the nuclear pellet and diluted to 20 mg/ml with dilution buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 2 mM ATP, 10 mM phosphocreatine, and 50 μg/ml creatine kinase). Extracts were stored at −80 °C or used immediately.

Nuclear protein was extracted as described (44). All steps were performed at 4 °C. Cells were incubated in 1.5 mM $MgCl_2$ for 30 min, pelleted at 300 × *g* for 10 min, rewashed in 1.5 mM $MgCl_2$, and repelleted. The nuclei were lysed in 0.6 M NaCl, 1 mM EDTA, 0.2% NaN_3, 5 μg/ml aprotinin, and 5 μg/ml leupeptin by gentle rocking for 1 h and microfuged for 1 h. The supernatant was dialyzed overnight against 20 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 0.02% NaN_3, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 11 μg/ml PMSF. The dialyzed solution was microfuged for 30 s, and the pellet was resuspended in 1 × SDS-PAGE sample loading buffer (31.2 mM Tris, pH 7.4, 1% SDS, 10% glycerol, and 2.5% β-mercaptoethanol).

Protein Electrophoresis and Microsequencing—hnRNP C proteins were resolved using 10% separating and 4% stacking SDS-polyacrylamide gel electrophoresis. For sequencing, nuclear proteins were resolved on a 10% slab (16 × 20 cm) gel. The gel was stained in 0.25% Coomassie Brilliant Blue, 10% glacial acetic acid, and 40% methanol and destained in 10% glacial acetic acid and 40% methanol. The bands of interest were excised, swollen in 1 × SDS-PAGE sample loading buffer, and concentrated by re-electrophoresis on an SDS-PAGE mini-gel (Bio-Rad). The protein was then electroblotted to ProBlott polyvinylidene difluoride using Towbins buffer (125 mM Tris-HCl, 95 mM glycine, 0.02% SDS, and 20% methanol). The polyvinylidene difluoride membrane was stained in 0.2% Ponceau S and 1% glacial acetic acid, and the piece of membrane containing the protein was excised and placed in a microfuge tube. Another piece of blotted membrane of similar size was excised as a background control and placed in a separate tube. The membranes were washed extensively in milliQ water and then incubated overnight in 50 μl of digestion buffer (1% reduced Triton X-100, 10% acetonitrile, 100 mM Tris, pH 8.0, and 0.2 μg of endoproteinase Lys-C). The membranes were sonicated in a water bath for 5 min and then microfuged for 5 min. The supernatant was transferred to a clean tube. Consecutive washes with 50 μl of digestion buffer and 100 μl of 0.1% trifluoroacetic acid were performed with sonication and centrifugation as described above. All 200-μl supernatants were pooled and stored at −20 °C until separation of the peptides by HPLC.

The HPLC system consisted of two ICI LC1100 pumps controlled using Hewlett-Packard Chemstation software and a Rheodyne injector. A narrow bore SynChropak RP-P (100 × 2.1 mm inside diameter) C_{18} column was equilibrated in 99% buffer A (0.08% trifluoroacetic acid in Millipore reverse-osmosis-purified water) and 1% buffer B (0.05% trifluoroacetic acid in acetonitrile) at a flow rate of 0.15 ml/min for 1 h. The 200-μl sample was injected into the column and eluted over a gradient of 1–30% buffer B (0–63 min), 30–60% buffer B (63–95 min), 60–80% buffer B (95–105 min), 80% buffer B (105–117 min), and 80–1% buffer B (117–127 min). The column was re-equilibrated in 1% buffer B for 1 h before loading the next sample. Peptides were detected using a Hewlett-Packard 1040A photodiode array detector collecting data at 220 nm with a reference wavelength of 350 nm. The blank was run on the same day as the peptide-containing sample, and fractions were collected by hand. Traces from the blank and sample were compared, and fractions corresponding to peaks present only in the sample trace were sequenced. Amino-terminal sequencing was performed on a Hewlett-Packard G1005A automatic sequencer.

Western Blotting—For Western blotting, equal quantities of total cell lysate were loaded per lane of a 10% slab gel. The gel was then electroblotted to Hybond-C nitrocellulose using Towbins buffer (50 V for 2 h). The membrane was washed in PBS for 1 h then blocked in 5% skim milk powder and PBS for 1 h. Primary antibody (4F4 for C protein and 9H10 for A1 protein) at a dilution of 1:1000 in 5% skim milk powder and PBS was added and rocked gently overnight at 4 °C. The membrane was then washed in PBS (3 × 20 min), and the horseradish peroxidase-conjugated anti-mouse Ig (Silenus; 1:1000) in 5% skim milk powder and PBS was added and rocked at room temperature for 2 h. The membrane was washed again in PBS (3 × 20 min). Proteins were detected using the DuPont Renaissance chemiluminescence kit with Reflection film.

Purification of hnRNP C Proteins—1×10^9 confluently growing BL30K cells were pelleted and lysed in cold 1.5 mM $MgCl_2$ (30 ml of $MgCl_2$/50 ml of cells) for 30 min at 4 °C. The cells were pelleted and washed in 1.5 mM $MgCl_2$ again for 10 min and repelleted. A small volume of resuspension buffer (90 mM NaCl, 1 mM $MgCl_2$, and 10 mM

Tris, pH 8.0) was added to each pellet, and the nuclei were combined into one tube. The nuclei were centrifuged at 500 × *g* for 10 min. 1 ml of nuclei was resuspended in 2 ml of resuspension buffer and sonicated (3 × 10 s with 15 s between bursts) on ice. The cells were checked for complete disruption and incubated for 20 min at 37 °C to allow free RNases to digest the RNA. The nuclear sonicate was centrifuged at 8,000 × *g* for 10 min at 4 °C in a Corex tube. The opalescent supernatant was loaded onto two 15–30% sucrose gradients (dissolved in resuspension buffer) and centrifuged at 25,000 rpm in an SW 28.1 centrifuge. Fractions of 500 μl were collected, and samples were run on SDS-PAGE to locate the hnRNP particles. These fractions were pooled and adjusted to 350 mM NaCl, 1 mM DTT, and 10% glycerol. The pooled hnRNP fraction was then loaded at 0.5 ml/min onto a MonoQ column pre-equilibrated with 280 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, and 1 mM DTT. The proteins were eluted with a 27-min linear gradient from 280 to 600 mM NaCl. The hnRNP C proteins eluted at about 450 mM NaCl. 0.5-ml fractions were collected and assayed for the presence of hnRNP proteins by dot blot Western analysis and by SDS-PAGE for purity. The preparation was highly enriched for hnRNP C proteins; however, it did contain a major contaminant of approximately 58 kDa and several higher molecular mass proteins in low amounts.

Cleavage of hnRNP C Proteins in Vitro by Recombinant ICE-like Proteinases—pGEX plasmids encoding ICE, Mch2α, Tx, CPP32, and Nedd2 have been described previously (24, 28, 45). Mch3α was cloned into the pET-21b expression vector (Novagen). Bacterial lysates were prepared by inducing bacteria carrying the plasmids with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3–6 h followed by lysis using sonication in a buffer containing 25 mM HEPES, pH 7.5, 5 mM EDTA, 2 mM DTT, and 0.1% CHAPS. The lysates were centrifuged for 10 min at 16,000 × *g*, and the clear bacterial extracts were collected.

Bacterial lysates were assayed for enzyme activity by incubation with *Z*-Asp-Glu-Val-Asp-(7-amino-4-trifluoromethyl coumarin) (final concentration, 50 μM) following the release of 7-amino-4-trifluoromethyl coumarin with time by spectrofluorimetry (excitation at 400 nm and emission at 505 nm) as described previously (46).

For cleavage of purified hnRNP C proteins *in vitro*, 17 μl of active lysate, 2 μl of 50 mM DTT, and 1 μl of purified C protein were incubated for 1 h at 37 °C, the reactions were terminated with the addition of 5 μl of 5 × concentrated loading buffer, and the samples were electrophoresed and Western blotted. As a control the bacterial lysate was replaced with 17 μl of bacterial lysis buffer.

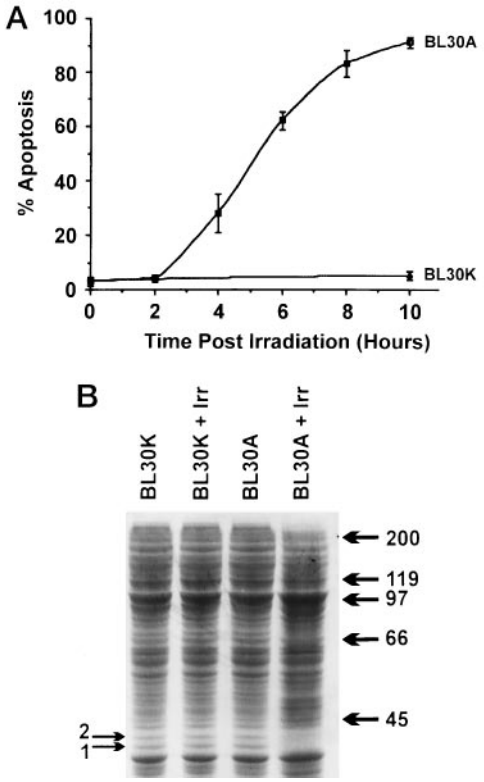

FIG. 1. *A,* induction of apoptosis in BL30A and BL30K cells by 20 Gy of ionizing radiation. The percentage of apoptotic cells with time after irradiation was determined by counting cells stained with Hoechst 33258. *Bars,* S.D. *B,* polyacrylamide gel electrophoresis of apoptotic and nonapoptotic cell extracts. A Coomassie Blue-stained 10% SDS-PAGE gel shows the disappearance of two ~40-kDa molecular mass proteins in the radiation-sensitive BL30A cells 8 h after irradiation (*Irr,* 20 Gy). These proteins remain intact in the resistant BL30K cells after irradiation.

RESULTS

hnRNP C Proteins Are Cleaved in BL30A Cells after Irradiation but not in a Radiation-resistant Subline, BL30K—In the search for potential new substrates for ICE-like proteases cleaved during apoptosis, we compared protein patterns on SDS-PAGE gels from a pair of isogenic Burkitt's lymphoma cell lines, BL30A and its subline BL30K. The BL30A line is sensitive and the BL30K line is resistant to induction of apoptosis by ionizing radiation. The characteristics of these cells, previously termed BL30 and BL30(s), respectively, have been described (42). The apoptotic response of the two cell lines to 20 Gy of ionizing radiation is shown in Fig. 1*A*.

BL30A and BL30K cells were irradiated with 20 Gy and harvested at 8 h, at which time 80–90% of BL30A cells were undergoing apoptosis, whereas BL30K cells showed less than 5% apoptosis (Fig. 1*A*). Nuclear extracts of the cells were separated by SDS-PAGE and stained with Coomassie Blue (Fig. 1*B*). It is evident from these gels that the majority of cellular proteins remained intact during the process of apoptosis in both cell types, but careful examination of the pattern from the sensitive line showed two prominent bands of approximately 40 kDa, which had disappeared by 8 h after irradiation (Fig. 1*B, lanes 3* and *4*). These proteins remained intact in the resistant line under these conditions (Fig. 1*B, lanes 1* and *2*). The identity of one of the 40-kDa proteins (protein 1, Fig. 1*B*) was established by *in situ* proteolytic digestion with endoproteinase Lys-C and sequencing the resulting peptides after HPLC purification. The HPLC profile obtained by digestion of protein 1 is shown in Fig. 2*A*. After comparison with the blank trace, three peptides (peptides 1–3) were selected for amino-terminal sequence analysis. All three gave sequences identical to the published sequence for hnRNP C1 and C2, as shown in Fig. 2*B*. These two proteins arise from alternate splicing with C2 differing from C1 by the insertion of 13 amino acids (Fig. 2*B, shaded*; Ref. 47). We conclude from this that protein 2 (Fig. 1*B*) corresponds to hnRNP C2.

To confirm the cleavage of these proteins during apoptosis we used Western blotting with a monoclonal antibody (4F4) specific for hnRNP C1 and C2. Fig. 3 shows an immunoblot of BL30A extracts at 0, 2, 4, 6, and 8 h after irradiation and BL30K extracts at 0 and 8 h. Once again there was no change in the hnRNP C proteins in the resistant cell line; however, the antibody clearly detected degradation products in the sensitive line, corresponding to the onset of apoptosis at 4 h (Fig. 1*A*). From the immunoblots it would appear that the cleavage is occurring in two steps, as evidenced by the doublets at 6 h. The change in molecular mass of the proteins is quite small, indicating that a short segment is removed from one end.

Cleavage of hnRNP C Proteins Occurs in Several Cell Types in Response to a Variety of Apoptotic Stimuli—Fig. 4*A* shows the cleavage of hnRNP C proteins in three further cell lines (U937, Molt-4, and BM13674) in response to ionizing radiation. In all cases degradation was apparent. These proteins remained intact in another radiation-resistant line, BL29 (Fig. 4*B*).

Other apoptotic stimuli in addition to ionizing radiation were used with BL30A cells to assess the universality of hnRNP C cleavage in apoptosis. As shown in Fig. 5, treatment of cells with etoposide, C8 ceramide, tetrandrine, and EGTA all resulted in the same cleavage pattern seen after irradiation. In the case of EGTA the degradation was incomplete, as these

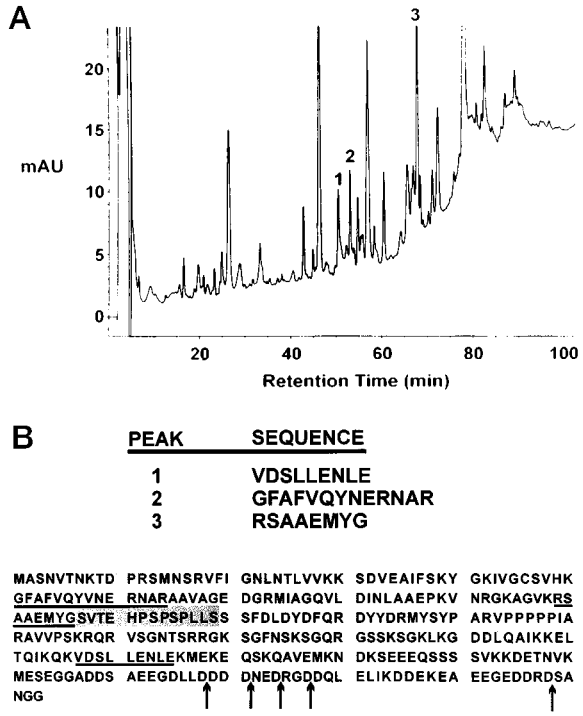

FIG. 2. **Identification of the 40-kDa proteins.** *A,* chromatogram of peptides generated from one of the ~40-kDa proteins by digestion with endoproteinase Lys-C, separated by narrow bore reverse phase HPLC. The absorbance was monitored at 200 nm. *Peaks 1–3* were sequenced. *B,* sequences obtained (*underlined*) from peaks 1–3 showed 100% identity to hnRNP C1 and C2 proteins. The 13-amino acid sequence absent in HNRNP C1 is *shaded*, and *arrows* show possible cleavage sites for ICE-like proteases.

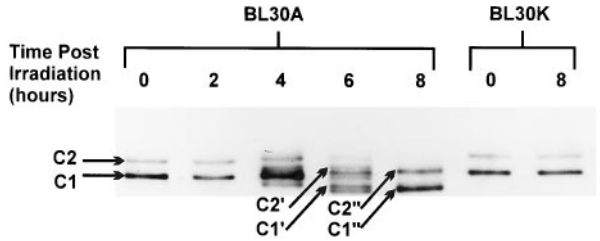

FIG. 3. **Western blot of total protein extracts from sensitive BL30A and resistant BL30K cells at the indicated times after irradiation with 20 Gy of ionizing radiation.** Primary antibody (4F4) to the hnRNP C proteins was used to show the specific cleavage of the C proteins after irradiation in BL30A but not BL30K cells.

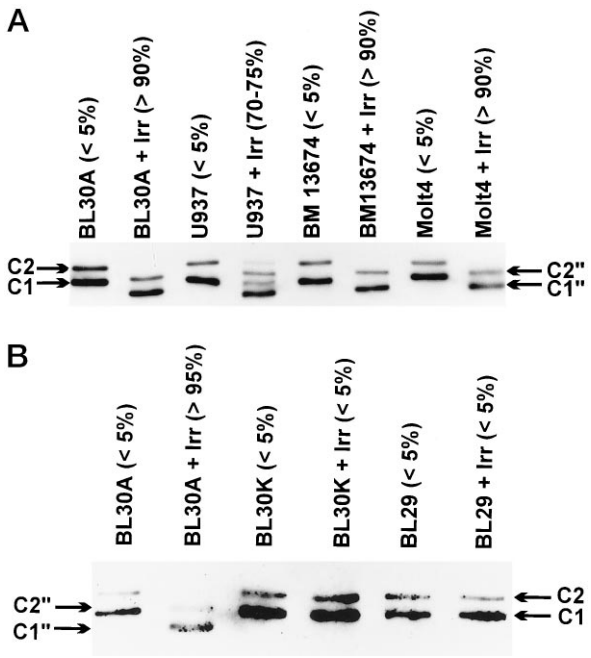

FIG. 4. **Western blot analysis of cell extracts from sensitive and resistant cell lines before and 12 h after irradiation with 20 Gy using 4F4 monoclonal antibody.** *A,* radiation-sensitive cell lines. *B,* radiation-resistant cell lines compared with BL30A. The percentage apoptosis as measured by Hoechst stain in each case is indicated in *parentheses*. *Irr,* irradiation

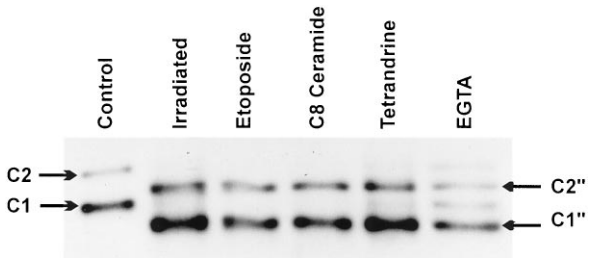

FIG. 5. **Cleavage of hnRNP C proteins in BL30A cells exposed to various apoptotic stimuli.** Cells were treated with irradiation (20 Gy), etoposide (68 μM), C8 ceramide (50 μM), tetrandrine (20 μg/ml), or EGTA (5 mM). All cells were harvested 12 h after treatment. The extent of apoptosis was 90–95% in all cases except EGTA treatment (70–75%).

cells are not as sensitive to apoptosis induced by this compound (only 70% of cells were apoptotic compared with 90–95% with the other treatments).

Cleavage of hnRNP C Proteins Is Mediated by ICE-like Proteases—To determine the protease involved in the cleavage of hnRNP C1 and C2 we irradiated BL30A cells in the presence of various protease inhibitors. As can be seen in Fig. 6*A*, the cleavage is inhibited by TPCK and TLCK but not by aprotinin, leupeptin, PMSF, and pepstatin. Fig. 6*B* shows that DNA degradation is also inhibited in the presence of TPCK and TLCK, and the extent of apoptosis in these cells was less than 5% as determined by Hoechst staining. Cells treated with the cell-permeable ICE inhibitor VAD-FMK also showed no degradation of hnRNP C or DNA laddering (Fig. 6*C*). These inhibition characteristics are consistent with those of the ICE family of cysteine proteases. We therefore extended our studies *in vitro* using DEVD-CHO and YVAD-CMK as inhibitors. The former is a very potent inhibitor of CPP32, whereas the latter is more potent for enzymes most closely related to ICE itself (23). The cytosolic fraction of apoptotic BL30A cells was added to untreated total cell extracts in the presence of various concentrations of DEVD-CHO and YVAD-CMK and the extent of cleavage of the hnRNP C proteins determined. As can be seen in Fig. 7, the proteolysis of hnRNP C was almost completely inhibited by 10 nM DEVD-CHO, whereas 10 μM YVAD-CMK was required to achieve the same effect. This is consistent with the cleavage being mediated by CPP32 or a close relative rather than by an enzyme more closely related to ICE. HnRNP C1 and C2 were not detected in the cytoplasm-only lane, since these proteins are confined to the nucleus.

Using this *in vitro* system we were never able to obtain complete cleavage of hnRNP C, unlike that observed in the whole cell experiments. However, under the same conditions complete degradation of DNA-PK was observed (Fig. 7*B*). The reasons for this are not clear at this stage.

Recombinant CPP32 was added to purified hnRNP C *in vitro*, and the cleavage pattern obtained was identical to that *in vivo*, except that once again incomplete cleavage was observed (Fig. 8*A*). This could not be overcome by the addition of more enzyme

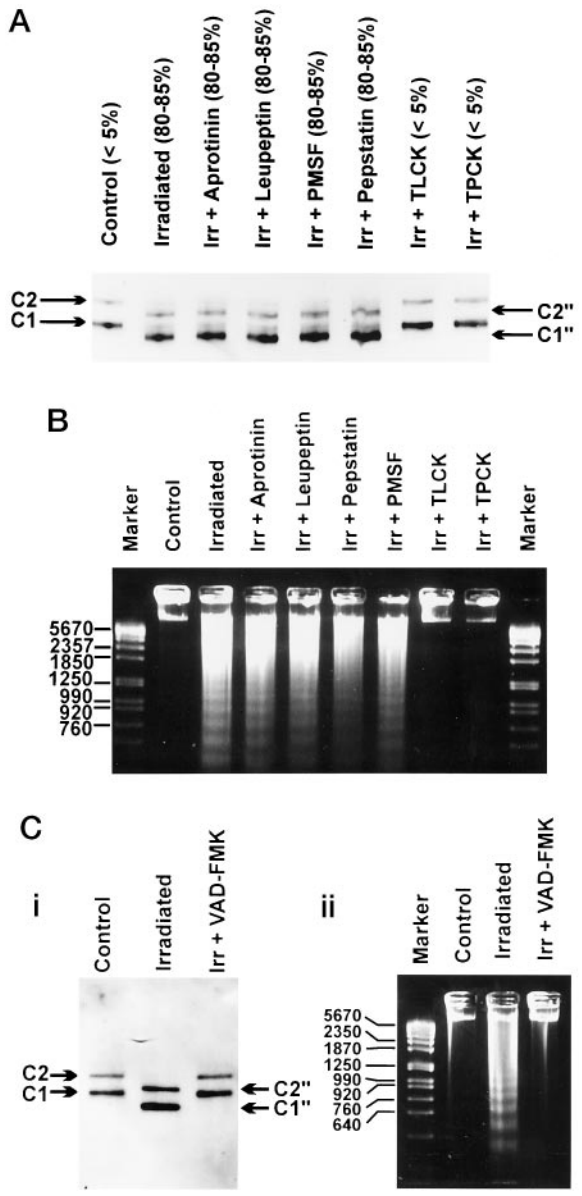

FIG. 6. **Effect of protease inhibitors on apoptosis and the cleavage of hnRNP C proteins in BL30A cells.** *A,* cells were irradiated (*Irr*) with 20 Gy 0.5 h after addition of various inhibitors: TPCK (200 μM), TLCK (400 μM), aprotinin (0.2 μM), leupeptin (210 μM), PMSF (570 μM), and pepstatin (3.6 μM). Cells were harvested for Western analysis at 8 h. The extent of apoptosis is indicated in (*parentheses*). *B,* inhibition of apoptosis by TPCK and TLCK as assessed by DNA laddering. *C,* VAD-FMK (10 μM), a cell-permeable specific inhibitor of the ICE-like proteases, also protects hnRNP C proteins (*i*) and DNA (*ii*) from degradation *in vivo*.

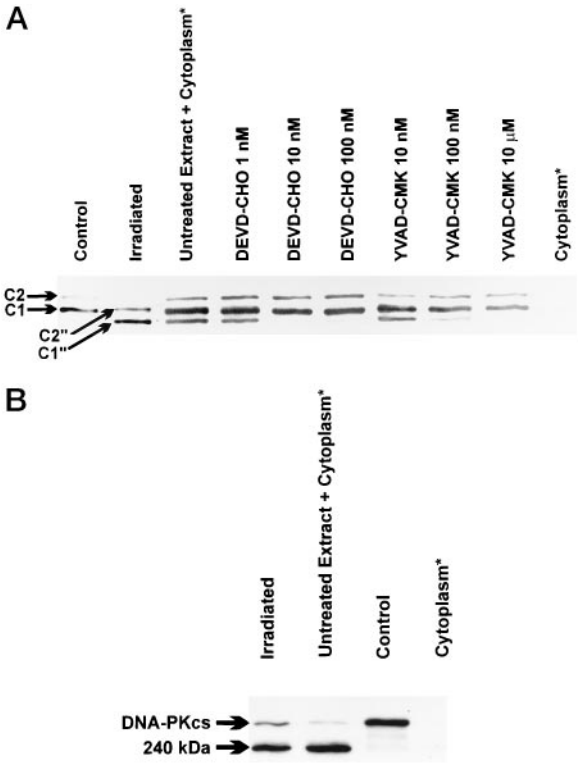

FIG. 7. **Inhibition of hnRNP C cleavage by inhibitors of ICE-like proteases *in vitro*.** *A,* cytoplasmic extracts (15 μg) from BL30A cells undergoing apoptosis were incubated with untreated total cell extracts (20 μg) for 1 h at 37 °C in a 20-μl reaction volume prior to separation by SDS-PAGE. *, cytoplasm from apoptotic cells. The inhibitors were added to the cytoplasmic extracts 30 min prior to incubation with the total untreated extracts. *B,* parallel samples were analyzed for degradation of DNA-PK using the DPK1 antibody as described by Song *et al.* (26).

during the incubation period or by extending the incubation to 4 h. The cleavage by CPP32 could be inhibited by the addition of *N*-ethylmaleimide or iodoacetamide (50 mM; data not shown).

To determine which enzyme is responsible for the cleavage of hnRNP C in apoptosis, we incubated purified hnRNP C proteins with seven recombinant ICE-like enzymes. Mch3α, CPP32, and to a lesser extent Mch2α cleaved both the C proteins, producing a pattern of degradation similar to that seen *in vivo*, whereas ICE, Tx, Nedd2, and granzyme B were unable to cleave the C proteins (Fig. 8*B*).

Since there are about 20 major proteins in the hnRNP complex (48), it was of interest to determine whether any others were also degraded during apoptosis. Using a monoclonal antibody (9H10) to hnRNP A1 and Western blotting, we were unable to detect any cleavage of this protein (data not shown).

DISCUSSION

The importance of the ICE family of cysteine proteases in the execution phase of apoptosis has recently been demonstrated, and a limited number of substrates for these enzymes have been described (11, 20, 49). It is not yet clear whether the various ICE family members represent redundant enzymes with overlapping functions or whether they are members of a proteolytic cascade with each enzyme having a specific subset of targets. The identification of new substrates and the ICE family members responsible for their cleavage will provide further insight into the molecular mechanisms of apoptosis.

In this study we used as a model system a pair of isogenic cell lines, one of which is resistant to apoptosis induced by ionizing radiation, to identify novel substrates for the proteolytic enzymes activated during apoptosis. Two proteins, identified as being degraded in the sensitive but not the resistant line after irradiation, were sequenced and shown to be identical to hnRNP C1 and C2. The degradation of these proteins was seen in a number of cell types and in response to a number of different apoptosis-inducing stimuli and was not seen in cell lines resistant to induction of apoptosis. The cleavage of the hnRNP C proteins was inhibited *in vivo* by TPCK, TLCK, and VAD-FMK, and *in vitro* by iodoacetamide, *N*-ethylmaleimide, and YVAD-CMK. No inhibition was observed with the protease inhibitors aprotinin, leupeptin, PMSF, and pepstatin. These inhibition characteristics are consistent with the involvement of an ICE-like protease. The cleavage of hnRNP C was almost completely inhibited by 10 nM DEVD-CHO but not by 10 nM YVAD-CMK, indicating the involvement of a CPP32-like enzyme. In the longer exposures of Fig. 7*A* we still see some

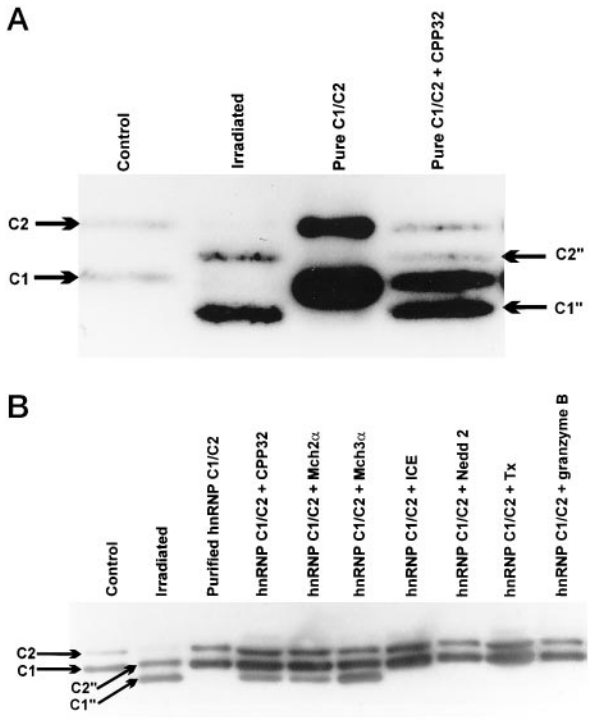

FIG. 8. **Cleavage of purified hnRNP proteins by recombinant ICE-like enzymes.** *A,* cleavage by CPP32. *B,* hnRNP C proteins digested with seven enzymes. The enzymes were assayed for proteolytic activity as described under "Experimental Procedures," and the CPP32 used in this experiment had one-half the activity of the others. The *center band* is in fact two bands, consisting of the cleavage product of C2 and the undigested hnRNP C1, shown more clearly in *A*.

cleaved product with 10 nM DEVD-CHO, suggesting that CPP32 itself may not be the optimal protease cleaving hnRNP C. As Mch3α has a much higher K_i for this inhibitor (28), this enzyme, or a closely related protease, may be the preferred enzyme for cleavage of hnRNP C *in vivo*. Using purified hnRNP C proteins and recombinant ICE-like enzymes *in vitro,* we demonstrated cleavage similar to that occurring *in vivo* by Mch3α, CPP32, and to a lesser extent Mch2α, but not by ICE, Nedd2, or Tx. Since ICE rel-III (15) is closely related to ICE, it is unlikely that this would cleave hnRNP C. The cytotoxic lymphocyte-specific serine protease granzyme B also failed to cleave the hnRNP C proteins. Granzyme B has recently been shown to be upstream of CPP32, since it cleaves the CPP32 precursor to its active form (50–52). It is therefore not surprising that this enzyme fails to cleave hnRNP C. Although not tested in this study, the recently discovered member of the ICE family, Mch4, lies upstream of CPP32 and Mch3 (19) and is thus unlikely to be a candidate for the hnRNP C-cleaving enzyme. From this and the evidence provided in this study we conclude that the likely mediators of hnRNP C cleavage *in vivo* are Mch3α and CPP32. It is possible that more than one enzyme is responsible *in vivo*, as evidenced by the intermediate products C2′ and C1′ (Fig. 3), although it is equally possible that the same enzyme could cleave twice. We have been unable to obtain complete cleavage using *in vitro* systems of apoptosis (adding cytosol from apoptotic cells to untreated cell extracts or recombinant enzymes to semipurified hnRNP C proteins), even though under the same conditions complete cleavage of DNA-PK was observed. It is possible that the cleaved products are inhibiting the enzyme, although no further cleavage was obtained by adding additional enzyme during the incubation. hnRNP C may need to be bound to other proteins *in vivo* as part of the hnRNP complex to be cleaved. In the process of preparing a cell extract these interactions may be disrupted to some extent, preventing complete cleavage. A full explanation of this phenomenon must await further experimentation.

As evidenced by the Coomassie Blue staining pattern, most proteins remain intact even when the majority of cells are undergoing apoptosis; thus a small number of critical proteins are cleaved to ensure death. hnRNP C1 and C2 can now be added to this list of critical targets.

The hnRNP C proteins belong to a class of proteins that bind heterogeneous nuclear RNAs (hnRNAs or pre-mRNA). There are at least 20 major proteins in the hnRNP complex or spliceosome (48). The hnRNP proteins are thought to play a role in processing pre-mRNA; however, their full range of functions and mechanisms of action are not yet known. hnRNP proteins bind RNA directly and have a modular structure with one or more RNA binding modules (RNP motif) and at least one other domain, mediating protein-protein interactions (47).

The hnRNP C proteins (C1 and C2) are abundant in the nucleus and bind poly(U) tenaciously (53, 54). C2 is identical to C1 except for a 13-amino acid insert, suggesting they are derived by alternate splicing. They contain an amino-terminal RNP motif and a carboxyl-terminal negatively charged segment, which contains a putative nucleotide (NTP) binding site and potential phosphorylation sites for casein kinase II (47). The C proteins are localized to the nucleoplasm and excluded from the nucleoli in interphase cells, and they become dispersed throughout the cell during mitosis. They do not shuttle in and out of the nucleus, as do other hnRNPs, *e.g.* A1 (55), which is not degraded during apoptosis (data not shown).

HnRNP C1 and C2 may participate in retention of incompletely processed pre-mRNAs in the nucleus, and high affinity binding sites for the C proteins have been mapped to nucleus-restricted sequences, namely the 3′-end of introns and sequences downstream of polyadenylation sites (53, 56). Antibodies to hnRNP C have been shown to inhibit splicing reactions *in vitro* (57). Both hnRNP C and A1 bind reiterated AUUUA sequences in the 3′-untranslated region or several mRNAs. These cis-acting sequences are important determinants in post-transcriptional regulation of gene expression, suggesting an additional role for hnRNP C and A1 in mRNA turnover and translation (58). HnRNP C proteins undergo phosphorylation by a casein kinase II-like enzyme (59), and several other kinases have been found in HeLa cells. One of these, (Cs kinase), caused a mobility shift of C proteins on SDS-PAGE (60). This kinase appears to be cell cycle-regulated, and the Cs proteins were most prevalent during mitosis.

Serine and threonine phosphorylation (via a casein kinase II-like nuclear kinase) has been shown to regulate the binding of C hnRNP proteins to pre-mRNA (61). Phosphorylation is required for binding to pre-mRNA, and a dynamic cycle of phosphorylation and dephosphorylation in spliceosome assembly has been proposed. Dephosphorylation is inhibited by okadaic acid (an inhibitor of Ser and Thr phosphatases 1 and 2A). The structure of the hnRNP RNA binding domain is very similar to that of U1 snRNP A (54), and interestingly, dephosphorylation of the 70-kDa protein of the U1 snRNP particle is required for an early precatalytic step in pre-mRNA splicing (62). This protein component of snRNP is also a substrate of CPP32 and is cleaved during apoptosis (31, 32). It is interesting to note that the hnRNP A1 and C1 proteins are more abundant than U1 snRNP.

The carboxyl-terminal portion of hnRNP is rich in aspartic acid residues, with five potential cleavage sites for ICE-like proteases, as shown by the *arrows* in Fig. 2. The function of this region could well be to serve as a substrate for proteolysis during apoptosis. The effect of cleaving a small portion off the end of the molecule on its activity remains to be determined,

since the RNA binding domain is at the amino-terminal end of the molecule. It is interesting that two components of the splicing complex have now been identified as substrates for ICE-like proteases. This strategy would ensure that energy is not wasted in the dying cell by processing messenger RNA that is no longer required for cellular functions. As stated above, the full range of activities of the hnRNP proteins have not been determined. As many of the hnRNP proteins bind single stranded DNA, they may also have roles in transcription, DNA replication, and recombination (48). The identification of hnRNP C1 and C2 as protease substrates in apoptosis and the high degree of conservation of these proteins in evolution suggest a critical cellular function. Elucidation of this function must await further experimentation.

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