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

Nigel Waterhouse‡§, Sharad Kumar¶, Qizhong Song‡, Phil Strike¶, Lindsay Sparrow¶, Gideon Dreyfuss**, Emad S. Alnemri‡‡, Gerald Litwack†‡, Martin Lavin§§, and Dianne Watters$$$ (Received for publication, August 9, 1996)

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 Mch2α and CPP32 and, to a lesser extent, by Mch2α, but not by ICE, Nedd2, Tx, or the cytosolic 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) 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-3, 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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‡ To whom correspondence should be addressed. Tel: 61-7-3362-0335; Fax: 61-7-3362-0106; E-mail: dianneW@qimr.edu.au.

1 The abbreviation used are: ICE, interleukin 1β-converting enzyme; PK, protein kinase; hnRNP, heteronuclear ribonucleoprotein; TPCK, i-1-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, Nα-p-tosyl-l-lysine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; YVAD-CMK, Tyr-Val-Ala-Asp-chloromethylketone; DEVD-CHO, Ac-Asp-Glu-Val aspartic acid aldehydes; PBS, phosphate-buffered saline; VAD-FMK, Z-Va-Ala-Asp-CH2F; PIPES, 1,4-piperazinediethanesulfonic acid; HPLC, high performance liquid chromatography; CPP32, cytochrome c 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.
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hnRNP C Proteins and Apoptosis

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. 1A.

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. 1A). Nuclear extracts of the cells were separated by SDS-PAGE and stained with Coomassie Blue (Fig. 1B). 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. 1B, lanes 3 and 4). These proteins remained intact in the resistant line under these conditions (Fig. 1B, lanes 1 and 2). The identity of one of the 40-kDa proteins (protein 1, Fig. 1B) was established by in situ proteolytic digestion with endoproteinase Lys-C and sequencing the resulting peptides after HPLC purification of the obtained digestion mixture (Fig. 1C). The sequence analysis of the peptides 1–3 was selected for amino-terminal sequence analysis. All three gave sequences identical to the published sequence for hnRNP C1 and C2, as shown in Fig. 2B. These two proteins arise from alternate splicing with C2 differing from C1 by the insertion of 13 amino acids (Fig. 2B, shaded; Ref. 47). We conclude from this that protein 2 (Fig. 1B) 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. 1A). From the immunoblots it would appear that the cleavage is occurring in two steps, as evidenced by the doublet 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. 4A 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. 4B).

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...
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 hnRNPC1 and C2 we irradiated BL30A cells in the presence of various protease inhibitors. As can be seen in Fig. 6A, the cleavage is inhibited by TPCK and TLCK but not by aprotinin, leupeptin, PMSF, and pepstatin. Fig. 6B 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. 6C). 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 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. HnRNPC1 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 and YVAD-CMK is required to achieve the same effect. This is consistent with the proteolysis being mediated by CPP32 or a close relative rather than by an enzyme more closely related to ICE. HnRNPC1 and C2 were not detected in the cytoplasm-only lane, since these proteins are confined to the nucleus.

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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. 8B).

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 cells resistant to induction of apoptosis. The cleavage of the hnRNP C proteins 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. 7A we still see some...
cleaved product with 10 nM DEVD-CHO, suggesting that CFP32 itself may not be the optimal protease cleaving hnRNP C. As Mch3a 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 Mch3a, CFP32, and to a lesser extent Mch2a, 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 CFP32, since it cleaves the CFP32 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 CFP32 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 CFP32 cleavage of hnRNP C in vivo are Mch3a and CFP32. 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 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 nucleusspecific 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 posttranscriptional 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 (58), 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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