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Mammalian Cell Death Proteases: A Family of Highly Conserved Aspartate Specific Cysteine Proteases

Emad S. Alnemri*

Department of Biochemistry and Molecular Pharmacology and Center for Apoptosis Research, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Abstract So far nine human aspartate-specific cysteine proteases (ASCPs) have been identified and cloned in our lab and others. Their sequence and structural homology to the nematode Ced-3 implicated them in the cell death pathway of mammalian cells. Recent evidence suggests that ASCPs initiate apoptosis by acting at or near the cell death effector level. However, it is not clear whether the activity of one or several of these enzymes is necessary for execution of apoptosis. In addition, it is not yet clear how the proenzymes of ASCPs are activated or what triggers their activation. Execution of apoptosis in higher eukaryotes is apparently more complicated than in nematodes. It is most likely that in mammalian cells this process involves the coordinated action of multiple ASCPs and multiple redundant proteolytic pathways. J. Cell Biochem. 64:33–42. © 1997 Wiley-Liss, Inc.

Key words: apoptosis; cysteine proteases; CPP32; Mch2; Mch3; Mch4; Mch5

Apoptosis is a fundamental biochemical process of selective and controlled elimination of cells within a multicellular organism during normal cellular differentiation and development [Ellis et al., 1991; Raff, 1992; Wyllie, 1980]. It is also involved in tissue homeostasis, aging, pathological processes, and irreversible cell injury [Ellis et al., 1991; Raff, 1992; Wyllie, 1980]. Emerging evidence suggests that apoptosis may be directly involved in many human degenerative diseases, autoimmune disorders, and neoplasia [Carson and Ribeiro, 1993; Carson and Tan, 1995; Fisher, 1994; Green et al., 1994; Martin et al., 1994; Thompson, 1995; Williams, 1991]. Misregulation or failure of apoptosis, for example, may result in development of cancer [Green et al., 1994; Thompson et al., 1992; Williams, 1991]. In addition, resistance to antitumor treatment could be attributed to insensitivity to apoptosis induction [Fisher, 1994]. Therefore apoptosis is a mechanism to be exploited when developing new chemotherapies for the treatment of cancer. As more is learned about the critical components regulating this mechanism, there will be new targets upon which to base chemotherapies. Significant progress in identifying critical components of programmed cell death came from genetic manipulations of the nematode *Caenorhabditis elegans* developmental death pathways [Ellis and Horvitz, 1986; Ellis et al., 1991]. These studies identified a set of genes, termed *Ced* genes (for cell death defective), that function in the developmental cell death pathway of the nematode. Mutations in three of these genes, *Ced-3*, *Ced-4*, and *Ced-9*, have been shown to affect the execution of cell death in all somatic cells that are destined to die. In dying cells, the activity of *Ced-3* and *Ced-4* is necessary for cell death to occur [Ellis and Horvitz, 1986; Yuan and Horvitz, 1990], whereas in surviving cells, the activity of *Ced-9* is required to block cell death [Hengartner et al., 1992]. The *Ced-9* gene product is homologous to mammalian *Bcl-2* protooncogene product known for its anti-cell death activity [Hengartner and Horvitz, 1994]. The mammalian counterpart of *Ced-4*, if any, has not yet been identified. Interestingly, the *Ced-3* gene product has been identified recently as a member of a growing family of ASCPs of which mammalian Interleukin-1 beta converting enzyme (ICE) is the best known example. Initial observations that ICE overexpression in mammalian cells causes apoptosis, implicated it as

*Correspondence to: Dr. Emad S. Alnemri, Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, Bluemle Life Sciences Building, 233 S 10th Street, Philadelphia, PA 19107.

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the mammalian equivalent of Ced-3. However, the recent identification and cloning of several other mammalian ASCPs that are homologous to ICE and Ced-3 suggest that one or more of these enzymes could be the key component of mammalian apoptosis. These enzymes, in particular CPP32, Mch2, and Mch3 [Fernandes-Alnemri et al., 1994, 1995a,b], the closest known relatives of Ced-3, will be prime targets for the development of agents that can activate these proteases and thereby the process of apoptosis. New homologs are being discovered, and it is not yet clear which enzyme will be the key one in the apoptotic program. Although ICE has been a major candidate, the absence of any significant effect on apoptosis in ICE-knockout mice [Li et al., 1995] suggests that there is yet another crucial enzyme(s) in this process. This enzyme, like other members of the family, is expressed as an inactive proenzyme awaiting a specific signal for its activation. The activation process itself might depend on the level of expression of this proenzyme in a given cell type and/or the expression of an upstream protease that is responsible for its activation. One way to approach this problem will be to determine which of the candidates is the actual molecular mediator of mammalian apoptosis, to understand the cellular processes(s) that activate this protease, to determine its relevant substrate(s), and to learn about the molecules involved in suppressing its activity. Here I will focus on our current knowledge of the mammalian Ced-3-like apoptotic ASCPs that were initially discovered and cloned in our lab, their structure, substrates, and role in apoptosis.

TABLE I. Human ASCPs Cloned From Jurkat T-Lymphocytes*

Cysteine protease	Apoptosis	% Identity to ICE	% Identity to CED-3	% Identity to CPP32
ICE		100	28	30
ICEα	+			
ICEβ	+			
ICEγ	+			
ICEδ	−			
ICEϵ	−			
Ich-1		28	28	29
Ich-11	+			
Ich-1s	−			
Ich-1β	−			
CPP32		30	35	100
CPP32α	+			
CPP32β	+			
Mch2		29	35	38
Mch2α	+			
Mch2β	−			
Mch3		26	33	53
Mch3α	+			
Mch3β	−			
Mch4		25	32	35
Mch4α	+			
Mch4β	+			
Mch5	?	25	34	41
Mih1		53	25	32
Mih1α	+			
Mih1β	+			
Mih1γ	+			
Mih1δ	+			

*ASCPs and variants listed in italics have been identified and cloned in our lab. (+) indicates ability to induce apoptosis. (−) indicates inability to induce apoptosis.

CLONING OF NOVEL MAMMALIAN ASCPS

An approach combining information from the GenBank database of human expressed sequence tags (ESTs) and PCR, was used to identify and clone several novel members of the family of CED-3/ICE-like apoptotic ASCPs from the human Jurkat T-cell line (Table I). As shown in Figure 1, human CED-3/ICE-like sequences present in a unidirectional Jurkat cDNA library are first enriched using degenerate PCR primers encoding the conserved GSWFI/GSWYI pentapeptides and a T3 vector specific primer [Fernandes-Alnemri et al., 1995a]. The enriched library is then amplified with a degenerate PCR primer encoding the conserved QACRG pentapeptide and SK vector specific primer located downstream of the T3 primer. The enriched library can be also amplified using primers derived from GenBank-ESTs representing potential ASCPs provided that the primers are located upstream of the GSWFI/GSWYI degenerate primers. This strategy ensures specific amplification of sequences encoding potential ASCPs. The products of the second amplification are cloned and sequenced to verify their nature. Clones that represent novel ASCPs are then used as probes to screen the original Jurkat cDNA library to obtain the full length cDNA clones. Using this approach we have cloned five novel apoptotic ASCPs named Mch2 [Fernandes-Alnemri et al., 1995a], Mch3 [Fernandes-Alnemri et al., 1995b], Mch4, Mch5, and Mih1 (Table I). Before this approach was developed we relied on RT-PCR and information from the GenBank-ESTs to clone CPP32 [Fernandes-Alnemri et al., 1994]. Phylogentically the fam-

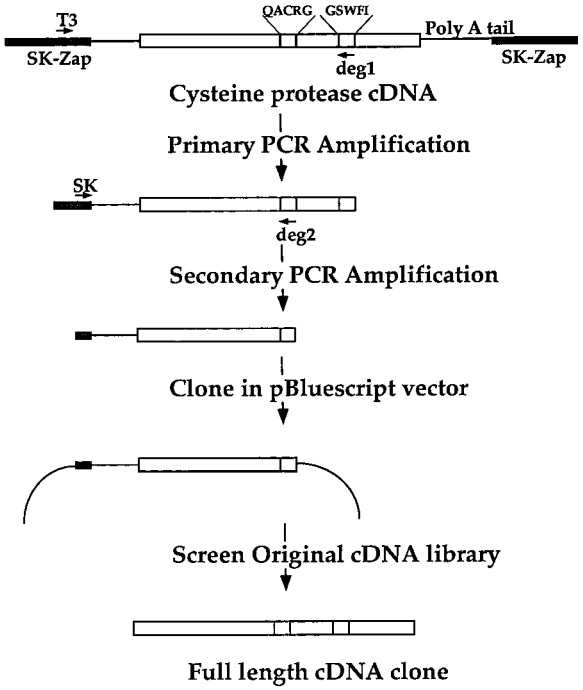

Fig. 1. Screening for novel Human ASCPs. A schematic diagram illustrating the degenerate primer-dependent PCR approach used to clone human apoptotic ASCPs. A unidirectional Jurkat cDNA library constructed in λ Uni-ZAP™ XR vector was used as a template for this PCR. Deg1 encodes the conserved pentapeptide GSWFI/GSWYI and deg2 encodes the active site pentapeptide QACRG. T3 primer: ATTAACCCTCACTAAAG. SK primer: CAGGAATTCGGCACGAG.

ily of ASCPs can be divided into three subfamilies. The Ced-3-like ASCP subfamily includes CPP32, Mch2, Mch3, and Mch4 and Mch5 (Table I). The ICE-like ASCP subfamily includes ICE, TX (ICH2, ICErel-II, Mih1), and ICE_{rel}-III [Faucheu et al., 1995; Kamens et al., 1995; Munday et al., 1995; Thornberry et al., 1992]. The NEDD-like subfamily includes ICH-1 and its mouse counterpart NEDD2 [Kumar et al., 1994; Wang et al., 1994].

CPP32

Human CPP32 is the closest relative of *C. elegans* Ced-3 based on overall amino acid sequence identity [Fernandes-Alnemri et al., 1994; Yuan et al., 1993]. Although other family members such as Mch2 and Mch3 are as closely related to Ced-3, it is widely believed that CPP32 is the most important mediator of apoptosis in mammalian cells. CPP32 mRNA encodes a polypeptide of 277 amino acids that represent the 32 kDa CPP32 precursor or proCPP32. ProCPP32 shows an overall 35% identity (58% similarity) with Ced-3 proenzyme. However, unlike Ced-3 proenzyme, it has a substantially short N-terminal propeptide. Based on the similarity between the secondary structure of proCPP32 and that of proICE (Fig. 2), we have predicted that Asp175 and Asp181 are potential processing sites in proCPP32 [Fernandes-Alnemri et al., 1994]. Cleavage at these two sites would generate two polypeptides equivalent to the p20 and p10 subunits of ICE. Analysis of the apoptotic activity of recombinant CPP32 subunits (p20, amino acids 1–175 and p11, 181–277) expressed separately or in combination demonstrated that the active CPP32 complex is in fact made up of two subunits derived from the proCPP32. This was later confirmed by mass spectroscopy and amino acid sequence analyses of purified human CPP32 [Nicholson et al., 1995]. In addition, it was demonstrated that the large subunit of CPP32 (amino acids 1–175) is further cleaved at Asp28 to remove a short N-terminal propeptide. Our data suggest that CPP32 can also undergo autocatalytic processing at Asp9 to generate a p19 large subunit. No cleavage was observed at Asp181, although a p11 subunit generated by expressing amino acids 181–277 is functional [Fernandes-Alnemri et al., 1994].

Northern blot analysis of human tissue RNA showed that CPP32 is widely expressed in all tissues examined. This suggests that apoptosis in most human cell types could be mediated by CPP32. The CPP32 gene was mapped to chromosome 4q34 [Bullrich et al., 1996].

Mch2

Mch is a short term for *m*ammalian *C*ed-3 *h*omolog. In human Jurkat T-cell line, two Mch2 transcripts were identified [Fernandes-Alnemri et al., 1995a]. A 1.7 kb transcript encodes a polypeptide of 293 amino acids that represent the ~34 kDa Mch2α precursor or proMch2α. A 1.4 kb alternatively spliced transcript encodes a 204-amino acid protein (Mch2β) that lacks approximately one-half of its putative p20 subunit and, therefore, is inactive. ProMch2α shows an overall 38% identity (56% similarity) with CPP32 and 35% identity (56% similarity) with Ced-3 proenzymes (Table I). The secondary structure of proMch2α is very similar to that of proCPP32. Like proCPP32, it has a substantially short N-terminal propeptide. Data on the expression and autoprocessing of proMch2α in bacteria suggest that Asp23 is the most likely processing site in the N-terminus of proMch2α.

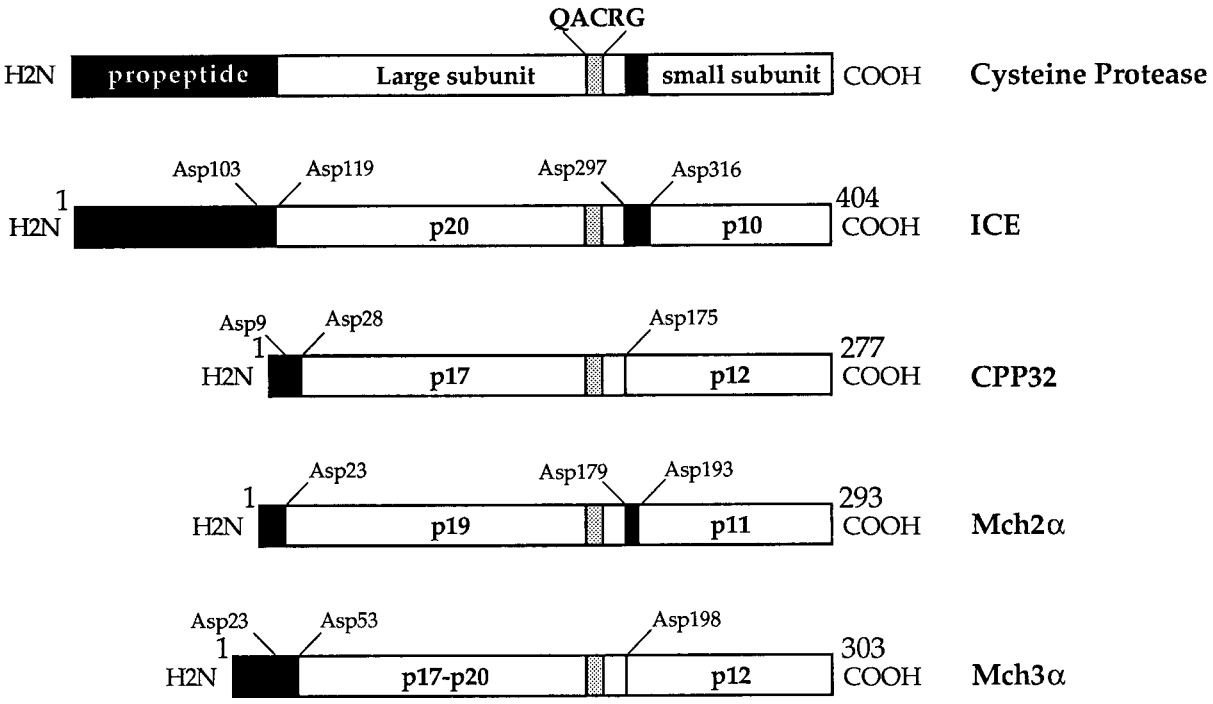

Fig. 2. Structure and organization of CPP32, Mch2, and Mch3 in relation to ICE. The propeptide domain and the linker region between the two subunits are shown as solid boxes. The QACRG active site pentapeptide is shown as a shaded box.

Potential processing sites between the two subunits of Mch2α are Asp179 and Asp193. Our data suggests that Asp193 is the most likely processing site in proMch2. Cleavage at these sites would generate two Mch2α subunits with calculated molecular masses of ~18–19 kDa (p19) and ~11 kDa (p11). These sizes are consistent with those obtained after processing of proMch2α in vitro. The Mch2 mRNA was also detected in a wide range of tissues and cell types. The Mch2 gene was mapped to chromosome 4q25 [Bullrich et al., 1996].

Mch3

Two Mch3 cDNA clones were isolated from human Jurkat T-cell line and named Mch3α and Mch3β [Fernandes-Alnemri et al., 1995b]. Mch3α cDNA encodes a 303 amino acid protein with a predicted molecular mass of ~34 kDa that represents the full length proMch3α. The second cDNA, Mch3β, arises from two simultaneous alternative splicing events that result in a deletion of exonic sequences and insertion of intronic sequences in the region that encodes amino acids 149–183 of Mch3α. As a result, Mch3β cDNA does not maintain the same reading frame as Mch3α after amino acid 148. Mch3β encodes a protein of 253 amino acids that lacks the conserved QACRG active site pentapeptide. The predicted full length proMch3α protein sequence shows the highest homology to human proCPP32. ProMch3α shares an overall ~53% identity (67% similarity) with proCPP32, compared to ~37% identity (55% similarity) with Mch2α and ~33% identity (55% similarity) with Ced-3 proenzymes (Table I). proMch3α shows less than 30% identity with the proenzymes of other family members such as ICE, NEDD/ICH-1, TX (ICH-2, ICE_{rel}-II) [Faucheu et al., 1995; Kamens et al., 1995; Munday et al., 1995] or ICE_{rel}-III [Munday et al., 1995]. In addition to the conservation of the active site QACRG pentapeptide, the organization of proMch3α appears to be similar to proCPP32 [Fernandes-Alnemri et al., 1994; Nicholson et al., 1995]. ProCPP32 is cleaved at Asp28 and Asp175 to generate two polypeptides with molecular masses of 17 kDa (p17) and 12 kDa (p12) that form the active CPP32 enzyme complex [Nicholson et al., 1995] (Fig. 2). Based on the high homology between proMch3α and proCPP32, the most probable cleavage sites in proMch3α are Asp53 and Asp198 (Fig. 2). Cleavage at these sites would generate two polypep-

tides equivalent to the p17 and p12 subunits of CPP32. However, the presence of a DSVD tetrapeptide (amino acids 20–23 of Mch3α) that is very similar to the DEVD tetrapeptide substrate of CPP32 [Fernandes-Alnemri et al., 1995a; Nicholson et al., 1995] suggests that it is the most likely processing site in the N-terminus of proMch3α. This suggests also that proMch3α might be a substrate for CPP32, as we describe below. Therefore, Mch3α is made of two subunits, a large p20 subunit and a small p12 subunit.

Northern blot analysis of human tissue RNA showed that Mch3 is widely expressed in all tissues examined. However, it is less abundant than CPP32 in brain tissues. The Mch3 gene was mapped to chromosome 10q25 [Bullrich et al., 1996].

Mch4 and Mch5

Mch4 and Mch5 were cloned by virtue of their high homology to CPP32 and Mch2. The organization of Mch4 and Mch5 proenzymes is similar to that of CPP32 except that they have a substantially longer N-terminal propeptide domain. Work is underway to fully characterize these proteases, their substrates and inhibitors.

Mih1

Mih is a short term for *m*ammalian *I*CE *h*omolog. The predicted amino acid sequence of proMih1 is identical to the amino acid sequences of TX (ICH-2, ICE_{rel}-II) [Faucheu et al., 1995; Kamens et al., 1995; Munday et al., 1995] described recently.

ACTIVITY AND SUBSTRATES OF MAMMALIAN APOPTOTIC CYSTEINE PROTEASES

To determine the activity of the various ASCPs, they were expressed in Sf9 cells using the baculovirus system. All of the above listed proteases were able to induce apoptosis when their proenzymes were overexpressed in Sf9 cells, suggesting that the proenzymes undergo correct processing in these cells. Although the baculovirus antiapoptotic protein p35 is an inhibitor of ASCPs [Bump et al., 1995] this inhibition is stoichiometric. Therefore, when ASCPs are overexpressed in Sf9 cells under the late polyhedrin promoter, the earlier virally encoded p35 is not sufficient to inhibit all the enzyme molecules which continue to accumulate and thereby induce apoptosis. Apoptosis can also be induced in Sf9 cells by cotransfection of the two physically separated subunits (large and small) of an ASCP. Interestingly, the ability of subunits derived from different family members to form functional heterocomplexes can be tested using this system. We have shown recently that the two closely related proteases CPP32 and Mch3α can form functional intermolecular heterocomplexes [Fernandes-Alnemri et al., 1995b]. Recombinant CPP32-p17 subunit can form an active heteromeric enzyme complex with recombinant Mch3α-p12 subunit and vice versa, as determined by the ability of the heteromeric complexes to induce apoptosis in Sf9 cells. In contrast, coexpression of CPP32 and ICE subunits in any combination does not induce apoptosis in Sf9 cells [Fernandes-Alnemri et al., 1994]. These observations suggest the possibility that Mch3α and CPP32 may heterodimerize in vivo in mammalian cells to form active proapoptotic complexes. These complexes may have specific activities different from those of the parental enzymes, adding to the complexity of the mechanism of apoptosis. To complicate things further, it is possible that the alternatively spliced isoforms of ICE (i.e., ICEϵ or ICEδ) [Alnemri et al., 1995] could form inactive antiapoptotic complexes with ICE or ICE-related proteases such as TX and ICE_{rel}-III. Other antiapoptotic complexes could be formed between the inactive Mch2β, Mch3β, or ICH-1s and closely related members of the family as well.

Table II lists all identified substrates of mammalian ASCPs. Although all these proteases

TABLE II. Mammalian ASCPs and Their Substrates

Protease	Substrate
ICE	Pro-IL1β, ProICE, ProTX, ProCPP32, PARP, U1–70 snRNP
NEDD-2/ICH-1	PARP
CPP32	PARP, DNA-PKcs, ProMch2α, ProMch3α, SREBP, U1–70 snRNP, ProCPP32
Mch2α	Lamins, PARP, proMch2α, U1–70 snRNP, ProCPP32
Mch3α	PARP, ProMch3α, U1–70 snRNP
Mch4β	PARP, proMch4β, U1–70 snRNP
Mch5	?
TX/Mih1	PARP, ProICE, ProTX, U1–70 snRNP
ICE_{rel}-III	?

can cleave poly (ADP-ribose) polymerase (PARP) [Lazebnik et al., 1994] and the synthetic PARP tetrapeptide DEVD-AMC substrate, CPP32 and Mch3α are the most efficient. Both Mch3α and CPP32 exhibit Michaelis-Menten kinetics in cleaving the DEVD-AMC substrate with *K*m values of 51 and 13 μM, respectively. Both enzymes are inhibited by the peptide aldehyde DEVD-CHO at low nM concentrations ($K_{iMch3\alpha}$ = 1.8 nM and K_{iCPP32} = 0.59 nM). In contrast, they are weakly inhibited by the ICE inhibitor peptide aldehyde YVAD-CHO (K_{iICE} = 0.76 nM, $K_{iMch3\alpha}$a > 10 μM and K_{iCPP32} = 8.5 μM) or the ICE inhibitor cowpox serpin, *Crm A* [Ray et al., 1992] ($K_{iMch3\alpha}$ > 1 μM and K_{iCPP32} = 0.56 μM). These observations confirm that the two enzymes, Mch3α and CPP32, are functionally similar to each other and may have similar substrate specificity. The high concentration of *Crm A* required to inhibit either CPP32 or Mch3α suggests that the target of *Crm A* inhibition in apoptosis is most probably not CPP32 or Mch3α. Further support for the functional similarity between CPP32 and Mch3α was obtained from studies using chimeric CPP32-Mch3α and Mch3α-CPP32 hybrid proteins. The CPP32-Mch3α chimera contains amino acids 1–175 (p17) of CPP32 fused in-frame to amino acids 199–303 (p12) of Mch3α. On the other hand, the Mch3α-CPP32 chimera contains amino acids 1–198 (p20) of Mch3α fused in-frame to amino acids 176–277 (p12) of CPP32. When the two chimeras were expressed in bacteria, we obtained functional active enzymes. The two chimeric enzymes can cleave the DEVD-AMC substrate and their ability to cleave PARP is similar to the parental enzymes. These findings support our earlier observation on the functional interchangeability of Mch3α and CPP32 subunits in induction of apoptosis in Sf9 cells [Fernandes-Alnemri et al., 1995b]. Furthermore, kinetic studies using the two chimeric enzymes could provide useful information on the contribution of the individual subunits of the parental enzymes to their substrate specificity. This can be done with substrates that are cleaved by one enzyme but not the other. We found that CPP32 can cleave the U1-70 kDa small nuclear ribonucleoprotein (U1-70 snRNP) [Casciola-Rosen et al., 1994] more efficiently than Mch3α (other members of the family can cleave U1-70 snRNP, although to a lesser extent) (Table II). The U1-70 snRNP can be used as a substrate for the kinetic studies with the chimeric and parental enzymes.

In addition to PARP and the U1-70 snRNP, other proteins such as DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [Song et al., 1995] and lamins [Lazebnik et al., 1995] are cleaved during cellular apoptosis. Interestingly, the sterol regulatory element-binding proteins (SREBP) has also been shown to be cleaved, but under normal physiological conditions, by CPP32 [Wang et al., 1995a].

The DNA-dependent protein kinase (DNA-PK) plays an important role in DNA double strand break repair [Hartley et al., 1995] and therefore it has an analogous function to PARP. In a collaborative study between our lab and Dr. Martin Lavin's lab (Queensland Institute of Medical Res., Brisbane, Australia), it was demonstrated that the DNA-PK catalytic subunit (DNA-PKcs) is preferentially degraded into three high molecular weight fragments after exposure of different cell types to a variety of agents known to cause apoptosis [Song et al., 1995]. This resulted in the loss of the DNA-PK activity. However, there was no effect on the DNA-binding component of the enzyme (Ku). Protease inhibitor studies suggested that an ASCP is responsible for the DNA-PKcs degradation. After exposure of purified DNA-PKcs to several ASCPs, only CPP32 was able to cleave this protein [Song et al., 1995]. The pattern of degradation in vitro by CPP32 resembled that observed in cells undergoing apoptosis. Furthermore, amino acid sequencing of one of the cleavage products identified a site of cleavage between Asp2709 and Asn2713. The sequence of this site is DEVD↓N which resembles the DEVD↓G site of PARP that is cleaved by CPP32. Therefore, CPP32 is the enzyme responsible for cleavage of both PARP and DNA-PKcs, which implicate CPP32 as one of the crucial components of the apoptotic cascade [Martin and Green, 1995; Whyte and Evan, 1995]. The physiological significance of PARP or DNA-PKcs degradation is not yet clear, in light of the observation that PARP deficient mice develop normally [Wang et al., 1995b]. However, the combined effect of losing the activity of two DNA repair enzymes, may have dramatic effects on the ability of the cell to repair their DNA and may accelerate the process of DNA degradation, which is a universal characteristic of apoptosis.

In a second collaborative study between our lab and Dr. William Earnshaw's lab (Institute

of Cell and Molecular Biology, University of Edinburgh, Edinburgh, Scotland, UK), it was demonstrated that the enzyme responsible for lamin cleavage is Mch2α and not CPP32 [Takahashi et al., 1995]. Mch2α not only cleaves PARP as we have shown recently [Fernandes-Alnemri et al., 1995a], it also can effectively cleave both human lamin A and lamin B [Takahashi et al., 1995]. The pattern of lamin degradation in vitro by Mch2α resembled that observed with S/M extracts and in apoptotic cells [Lazebnik et al., 1995]. Lamin degradation might be responsible for nuclear disintegration into small apoptotic bodies, a typical morphology observed during the late stages of apoptosis [Lazebnik et al., 1995].

ACTIVATION OF ASCPS

The mechanism of activation of ASCPs is not yet fully established. Recent observations suggest that activation of ASCPs in apoptosis occurs via a protease cascade. For example, TX can process proICE [Faucheu et al., 1995] and ICE can activate proCPP32 [Tewari et al., 1995]. Similarly, granzyme B, the cytotoxic T lymphocyte protease, which is essential for induction of apoptosis in target cells, can also cleave and activate CPP32 [Darmon et al., 1995]. Other ASCPs such as Mch2, Mch3, and Mch4 might also be activated by granzyme B. Furthermore, we have observed that CPP32 can cleave and activate proMch2α and proMch3α [Fernandes-Alnemri et al., 1995b]. Thus activation of TX, ICE, or CPP32 could lead to an amplifiable protease cascade, thereby activating apoptosis. Because there is evidence for the existence of at least nine human ASCPs, it is of fundamental importance to determine their interactions with each other. This should establish the molecular order of these proteases and identify which one of them is near the effector level.

DOES ALTERNATIVE SPLICING REGULATE THE ACTIVITY OF ASCPS?

Enzymatically inactive alternatively splice isoforms of ASCPs could regulate the enzymatic activity of the parental enzymes by acting as dominant negative inhibitors. Regulation of ASCP activity by alternative splicing suggests that this process may play a crucial role in the molecular mechanisms of apoptosis. We recently described several alternatively spliced isoforms of ICE [Alnemri et al., 1995]. Alternatively spliced variants of other ASCPs were also identified recently in our lab [Fernandes-Alnemri et al., 1994, 1995a,b]. CPP32β contains a deletion of most of its 5′ nontranslated region. Although this deletion does not affect its coding sequence it may have some effect on the stability of its mRNA. Other alternatively spliced CPP32 isoforms may also exist in different tissues at different developmental stages. Employing RT-PCR to analyze the expression of Ich-1 mRNA in the human Jurkat T-lymphocyte cell line we also identified a novel Ich-1 mRNA isoform which is equivalent to ICEδ isoform (Alnemri et al., manuscript in preparation). This Ich-1 isoform, termed Ich-1β, is different from Ich-1s described recently by Wang et al. [Wang et al., 1994]. This isoform contains a deletion downstream of the first potential Asp316 cleavage site between the large and small subunits. The deletion alters the coding frame and causes a termination 10 amino acids downstream of Asp 316 essentially generating a functional large subunit. Unlike Ich-1s isoform [Wang et al., 1994], we believe that Ich-1β isoform is a positive regulator of Ich-1 activity. Similar to the Ich-1s, the inactive Mch2β and Mch3β could be negative regulators of apoptosis and could inhibit the activity of the parental enzymes by acting as dominant inhibitors. Mch4β and the Mih1(TX) isoforms contain deletions in their propeptide domain and could still be processed to active enzymes. However, the effect of these deletions on the rate of activation of these enzymes is still unknown. Recent evidence suggests that the propeptide domain of ICE plays an important role in its activation [Ramage et al., 1995].

CONCLUSIONS AND PROSPECT

In the last several years apoptosis research has developed to become one of the most prominent and competitive areas of biological research. This is due in part to the potential for development of pharmaceuticals that could modulate this process which is intimately associated with several human diseases ranging from cancer to neurodegenerative disorders. Products of the newly discovered mammalian ASCP genes are excellent targets for development of such drugs. Ample evidence suggests that these ASCPs are essential for apoptosis execution. Nevertheless, it is not yet known whether all ASCPs play a role in mammalian apoptosis, or if it is a function of one or few of these enzymes. In addition, it is not known

what exactly triggers the conversion of inactive pro-ASCPs to active ASCPs when the cell enters the committed phase of apoptosis. One possibility is the existence of upstream cell death effectors or activators that interact directly with one or more of these pro-ASCPs or indirectly with components of the ASCP activation pathway. The availability of potential cell death genes like the ASCP genes from mammals, worms, and insects should facilitate the identification and isolation of interacting proteins and other components of the cell death pathway. Because the basic apoptosis program has been highly conserved during evolution, studies of the apoptosis effectors in lower organisms such as insects should contribute to a better understanding of their mammalian counterparts. Although ICE has been known for several years its mechanism of activation is not yet known. Under normal physiological conditions proICE is activated in monocytes and cleaves proIL-1beta to produce mature IL-1beta cytokine. Other downstream pro-ASCPs such as proCPP32 might also be activated by active ICE, nevertheless, the cells do not undergo apoptosis. This suggest that a threshold of tolerance for active ASCPs may exist in different cells depending on, among others, active enzyme concentration, substrates, endogenous inhibitors, and/or enzyme compartmentalization. The fact that the primary function of ICE is not apoptosis execution and there exist at least nine mammalian ASCPs expressed in a single cell type (i.e., human Jurkat T-lymphocytes), suggest that their primary function may not be apoptosis execution after all. The enzymatic activity of ASCPs might be required for as yet unidentified normal cellular processes. The participation of ASCPs in apoptosis execution may occur only after the threshold of tolerance is overcome by increased concentration of the active enzymes, diminished level of substrate or inhibitor, and/or decompartmentalization of the active enzyme, events that could occur under many cell death situations. Increased endogenous level of an active ASCP could lead to expansion of its substrate range (decreased substrate specificity) to include proteins that are not normally cleaved by that particular ASCP. This is evident from the ability of ICE to cleave PARP, or TX to cleave pro-IL-1beta, at high enzyme concentrations. Also overexpression of the proform of many of these enzymes in different cell types results in apoptosis. This decrease in substrate specificity could lead to cleavage of proteins essential for cell survival. Additionally, since all ASCPs are activated by cleavage after an Asp residue, an active ASCP could also cleave and thereby activate other family members resulting in an amplified protease cascade. Obviously, a lot of questions need to be answered before we know the exact function of each of these ASCPs. The identification and molecular cloning of several members of mammalian ASCPs should allow detailed biochemical, structural, and functional studies. Future studies such as targeted gene knockout studies, for example, should help to determine the function of these ASCPs and which one of them, if any, is the actual functional homolog of Ced-3. The outcome of such knockout experiments depends on whether apoptosis execution in vertebrates is an activity of one ASCP or requires the participation of several ASCPs, and whether one family member may substitute for another. Cell-free experiments suggest that several ASCPs are required for completion of apoptosis in vertebrates [Lazebnik et al., 1995]. Genetic complementation experiments have shown that CPP32 and Mch3, or TX and ICE are highly related in structure and substrate specificity, that subunits derived from one could compliment the other [Fernandes-Alnemri et al., 1995b; Gu et al., 1995]. Therefore, it is expected that knocking out one ASCP may not totally abrogate apoptosis in all cells, since many ASCPs are expressed in a tissue-specific manner. Consequently, there may be some redundancy within the family of ASCPs, although we believe that under normal physiological conditions each of these enzymes has a distinct function and a distinct subset of substrates. It is anticipated that these future studies will contribute to the elucidation of the mechanism of activation and function of this important family of proteases.

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