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Proteases in apoptosis

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Abstract. The interleukin-1β-converting enzyme (ICE)-like family proteases have recently been identified as key enzymes in apoptotic cell death. Among these proteases one can identify specific activities which may be involved in cytokine production or in resident protein cleavage. Several factors influence the constitutive apoptotic mechanism and may provide insight into the role of protease(s) in apoptosis. Although it appears that ICE family members play a most important role in promoting apoptotic cell death, evidence has been advanced that other proteases are also involved in sequential or parallel steps of apoptosis. Activation of a particular protease can lead to processing molecules either of the same or different proteases, leading to an activation of a protease cascade. Here we attempt to summarize the current thinking concerning these proteases and their involvement in apoptosis.
Key words. Apoptosis; proteases; ICE-like proteases; protein substrates.

Introduction

Early evidence for the role of protease activation in apoptosis came from observations made by Lockshin [1, 2] and Bowen [3] during programmed cell death in the intersegmental muscles of insects during metamorphosis. In these studies, the authors observed an elevation of autophagic lysosome activity in areas of active cell death. It is now generally believed that the release of acid hydrolases from lysosomes plays a secondary role in cell death, since in several different experimental systems lysosomes appear intact until the final stages of cell disruption. Later investigations into proteolytic mechanisms identified the granule exocytosis pathway of lymphocyte-mediated cytotoxicity and provided evidence that granule proteases, otherwise known as the granzymes [cytotoxic cell proteases (CCP)/fragmentins] contribute to the lethal damage inflicted upon target cells (see ref. 4 for review). Kaufmann [5] subsequently demonstrated that proteolysis of several nuclear substrates was an early characteristic of apoptosis in a human myeloid cell line exposed to various chemotherapeutic agents, and more recently Williams and Henkart [6] reported that several distinct proteases could induce the morphological and biochemical features of apoptosis upon their introduction into different cell types. Interest in the role of intracellular proteases during apoptosis was markedly stimulated by the observation that both the *Caenorhabditis elegans* cell death gene, *ced-3*, and its mammalian homologue, interleukin-1β-converting enzyme (ICE), contain a conserved pentapeptide domain at the active site and share a strong structural homology [7, 8]. Following this observation, additional members of the ICE gene family, with cysteine protease properties, have been cloned (*Ich-1/Nedd-2*; *Ich-2/TX/ICE$_{rel}$II*; *ICE$_{rel}$III*; *Mch2*; *prICE/CPP32/apopain/YAMA* and *Mch3/ICE-LAP3/CMH-1*) [9–19]. Overexpression of any one of these leads to apoptotic cell death, which is consistent with the conclusion drawn by Williams and Henkart [6]. Although it appears that ICE family members play a vital role in promoting apoptotic cell death, evidence has been advanced that other proteases are also involved in this process. Several articles have recently described the involvement of other proteases in experimental models of apoptosis. The following is our summary of the current thinking concerning these proteases and their involvement in apoptosis.

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Involvement of ICE in apoptosis

The seminal work by Gagliardini et al. [20] showed that microinjection of an ICE-cDNA expression vector into chicken dorsal root ganglion neurons results in cell death. Moreover, peptide inhibitors of ICE arrest the death of motor neurons following in vitro trophic factor deprivation during the period of naturally occurring cell death in vivo. Studies where animals were treated with ICE inhibitors demonstrated a maintenance of interdigital tissue which is normally lost during development. Increases in ICE activity have been reported in different cell types stimulated with agents that promote apoptosis, including Fas mAb. Transient expression of antisense ICE resulted in an inhibition of several types of apoptosis, but only a 50% reduction in Fas-mediated cell death [21]. Anti-Fas mAb rapidly stimulated the proteolytic cleavage of the test ICE-like substrate. Using the tetrapeptide inhibitor YVAD, two groups independently demonstrated a dose-dependent inhibition of the anti-Fas-initiated apoptotic process [21, 22]. How-

ever, it is necessary to point out that this and other inhibitors and substrates used in these studies would indicate not only the activity of ICE but also that of the ICE-like proteases [23]. This idea is supported by the finding that ICE-deficient mice exhibit few defects in apoptotic cell death and proceed through development normally, in contrast to *ced-3* mutants of *C. elegans* [24, 25].

A common feature among known ICE and ICE-related proteases is a highly restricted cleavage specificity for an aspartic acid P_1 residue. Such specificity suggests a role in protein processing rather than in general protein degradation [26]. This substrate specificity is somewhat unusual in that it is also shared by the cytotoxic granule protease, granzyme B, which is a serine protease. Granzyme B and ICE are inhibited by similar antagonists. Granzyme B, upon delivery to the cytosol of target cells, is sufficient to provide an apoptotic signal. Granzyme B may mimic ICE-like proteases upon introduction into target cells and activate resident cytoplasmic enzymes by proteolytic cleavage. However, a recent study showed that precursor interleukin (IL)-1β is not a substrate for granzyme B [27]. Instead, granzyme A has been shown to process pro-IL-1β. Although the site of cleavage differs, the processed and secreted IL-1β is biologically active [28]. As it turns out, in vitro activation of the apoptotic protease CPP32 by granzyme B has been demonstrated [27, 29, 30]. Since it is difficult to detect granzyme B in granule targeted cells, it has not been determined whether this enzyme will cleave and activate cytoplasmic CPP32 in vivo. The shared substrate specificity of ICE and granzyme B promoted a reasonable speculation, but it has now been shown that these two enzymes cleave different protein targets: the former converts pro-IL-1β into mature cytokine and the latter activates CPP32.

The only known substrate of ICE is pro-IL-1β (although rICE has been shown to cleave Bcl-x in vitro). Yet ICE expression has been observed in many cell types that do not appear to produce IL-1β, suggesting that there may be additional substrates for ICE. To test this hypothesis, macrophages stimulated with lipopolysaccharide (LPS) and adenosine triphosphate (ATP) or incubated with Cytotoxic Thymus-derived Lymphocytes (CTLs) were shown to undergo apoptotic changes in the presence or absence of the specific ICE inhibitor YVAD-cmk. No mature IL-1β cytokine was detected in either cell lysates or culture supernatant, confirming the successful inhibition of the primary ICE activity. The results imply that if additional ICE substrates exist in cells, they are not of critical importance in the progression of apoptosis and, at least in this system, YVAD-cmk can uncouple the processing of pro-IL-1β and apoptosis [31]. In other studies, secretion of IL-1b from monocytes, mediated by active ICE at the plasma membrane, occurs in the absence of demonstrable evidence of apoptosis. This suggests that enzyme activity coupled to IL-1β secretion does not trigger the apoptotic machinery [32]. Together, these data provide evidence that ICE by itself is not sufficient to induce apoptosis. This process is probably driven by other distinct ICE-like proteases which become active under different activation/regulatory controls.

Involvement of proteases other than ICE-like proteases in apoptosis

Several studies implicate other proteases in apoptosis. Calpain, a calcium-dependent neutral protease and a member of the papain family of cysteine proteases, degrades a number of important cellular proteins including proto-oncogenes, steroid hormone receptors, protein kinases and cytoskeleton proteins [33]. Calpastatin regulates the activity of calpains via allosteric interactions; the activity is decreased by comparable increases in cytosolic free calcium concentration needed to activate this calcium-dependent protease. A calcium requirement for different types of apoptosis is well documented [34], and changes in the intracellular free calcium concentration may also activate calpain at this time. Such an activation of calpain is observed in thymocyte apoptosis induced by diverse stimuli [35], in myocardial cell injury induced by hypoxia [36], in neuronal cell death in the brain during Alzheimer's disease [37] and in human monoblast U937 cells treated with tumour necrosis factor (TNF) and interferon (IFN)-γ [38].

Studies using calpain inhibitors have revealed that different activation conditions can result in either a critical [35], a partial [39] or an apparent lack [40] of protein target(s) through which calpain contributes to apoptosis. For example, treatment of human prostate adenocarcinoma cells with calpain inhibitors alone can induce apoptosis, suggesting that particular protein targets of calpain cleavage are involved in the induction of apoptosis [40]. Calpain inhibitors, on the other hand, cannot protect Jurkat cells treated with anti-Fas antibody, suggesting that a calpain-independent pathway is involved [41]. In yet another situation, U937 cell treatment with calpain inhibitors can block TNF- and cycloheximide-induced apoptosis [41] while accelerating TNF- and IFN-γ-induced apoptosis in the same cells [38]. These results suggest that one agonist triggers a pathway which involves calpain activation (under conditions which reveal inhibitor activity), but when used in combination with a second agonist this pathway may be bypassed and a route activated which involves calpain inactivation. Whether these differences reflect calpain isoform susceptibility to different inhibitors or are due to the presence of protein targets in differing cell types must await further studies and the emergence of a consistently clear picture.

Calpain is also known to play a key role in the processing of IL-1α [42]. Since the calcium ionophores A23187 and ionomycin enhance the processing and secretion of IL-1α and induce apoptosis in different cells, it was considered possible that calpain may also play a role in the processing of IL-1β. The activation of calpain neither enhances the processing of IL-1β by ICE in vitro or in vivo nor results in the degradation of precursor of IL-1β [43]. It is likely that calpain-mediated enhancement of IL-1α maturation and secretion is restricted to specific key events in a pathway, which functions independent of calcium-induced apoptosis. Based on a comparison of current apoptosis models, it is difficult to place calpain as a key enzyme in apoptosis induction. However, it is most likely that calpain plays some role in apoptosis, together with other cellular proteases.

A second Ca^{2+}-dependent serine protease, termed CRP, has also been implicated in apoptosis [44, 45]. This protease is associated with the nuclear matrix (and endoplasmic reticulum), where it may catalyse the cleavage of nuclear antigens in response to certain apoptotic triggers. Whether the activation of this protease is under the control of ICE-like proteases (or vice versa) is unclear, although the nuclear location of CRP makes it an attractive candidate for activation by an ICE-like protease.

The plasminogen activator system is an important proteolytic complex responsible for the breakdown of the extracellular matrix. It is known that plasminogen activator inhibitor type 2 (PAI-2) is a major product of monocytes and macrophages in response to inflammatory mediators. Recently, it has been shown that expression of PAI-2 protects against TNF-induced apoptosis in HeLa cells, a cell line that does not synthesize PAI-2 or contain significant levels of urokinase plasminogen activator (uPA) [46]. The mechanisms by which PAI-2 confers resistance of cells to apoptosis are not yet clear. In spite of a high degree of structural similarity between PAI-2 and crmA [46] it is unlikely that PAI-2 represents the mammalian homologue of crmA. While PAI-2 contains Arg at the P_1 position, crmA contains an Asp at this residue, which is recognized by the catalytic mechanism of ICE. A PAI-2 proteolytic cleavage product has been linked to apoptosis in myeloid leukemic cells, suggesting that a loss of the apoptotic inhibitory activity of PAI-2 is associated with proteolytic cleavage of the PAI-2 molecule. Intracellular PAI-2 might be an important factor in regulating apoptosis in certain systems through the inhibition of an as yet unidentified cell death protease.

Recently, soluble Fas ligand (sFasL/p27) has been detected in the cell culture supernatants of transfected COS cells (human FasL) and activated human peripheral T cells [47]. Several sFasL multimers were recovered, and it was determined that the ~70-kDa protein fractions contained biological activity, suggesting that the trimeric form of sFasL can effect FasR cross-linking. Because inhibitors of metalloproteases (MMP) affect the release of both pro-TNFα and TNF receptors [48, 49], it was considered possible that MMP activity might be involved in the generation of sFasL. It has been shown [50] that a specific MMP inhibitor (1,10 phenanthroline) increased the cell surface expression of FasL in activated and control Molt-4 cells. Culturing cells in the presence of $ZnCl_2$ reversed the effects of this inhibitor. The observed increases in FasL surface expression were independent of either PMA and ionomycin stimulation or protein synthesis. This suggests that these cells express FasL independent of stimulation and that MMP involvement occurs during the membrane expression of a presynthesized protein. By using a hydroamic-acid based series of MMP inhibitors in an FasL-overexpressing murine T-cell line, Kayagaki and associates [51] showed that these compounds appear to block the release of sFasL and favour the accumulation of FasL(p40/p45) at the cell surface. In a human T_h1 cell line (HML-1), PMA and ionomycin stimulated only a small increase in cytosolic FasL/p40. Most of the FasL in this response presented as an sFasL/p27 monomer in the cell lysate and culture supernatant. In this system, metalloprotease inhibitors increased the presence of cell lysate FasL/p40, while the sFasL/p27 in the supernatant was greatly reduced.

Normally, FasL expression is restricted to activated NK, T_h1 and CTL cells [52, 53]. The activity of metalloproteases attempts to ensure this restricted FasL expression by lowering the level of FasL in both T-cell lines and overexpressing transformants. In both systems the release of a consistently cleaved product (i.e. p27), which is blocked by metalloprotease inhibitors, suggests that the cleavage and uncoupling of FasL/p40 is MMP-based. In certain situations sFasL released from cells would inhibit target-specific Fas-mediated cytotoxicity by interfering with receptor ligation. Here, MMP inhibitors may provide a two-fold benefit by decreasing sFasL and by increasing the expression of FasL on activated effector cells.

Role of viral proteins in the regulation of protease activity

It appears that ICE is the only known protease which can be inhibited by crmA, a pox virus protein that apparently mediates the virus block of host-cell suicide. CrmA is a member of the serpin family and inhibits ICE by forming an active site-directed complex. CrmA might have antiapoptotic activity in addition to its ability to inhibit processing of IL-1β. Transfection of crmA into different cell types does confer resistance to apoptotic activity induced by CTL, anti-Fas antibody or TNF [21, 22, 54]. This inhibition by crmA was 'transfection' dose-dependent; a high level of crmA probably forms an overwhelming number of active site

complexes which can block apoptosis. Recently, evidence for the baculovirus protein p35 inhibition of ICE-like protease activity was shown in an enzymatic assay [55–57]. Inhibition of enzymatic activity correlated with the formation of a stable protease-p35 complex [56]. As with crmA, the co-expression of p35 gene product inhibits virally induced apoptosis, programmed cell death in *Drosophila*, neuronal cell death, and TNF- and Fas-mediated apoptosis. Unlike crmA, p35 has no effect on granzyme B [56]. Conversely, p35 has a specific inhibitory site for the CED-3 protease, whereas crmA does not [57].

Two other viruses with human trophisms have been shown to inhibit apoptosis. BHRF1, an Epstein-Barr virus gene product, and adenovirus E1B 19 kDa protein confer cellular resistance to apoptosis induced by diverse stimuli [58, 59]. BHRF1 and Bcl-2 are functionally and mechanistically similar. E1B 19 kDa and Bcl-2 are structurally similar, and E1B 19 kDa alone is sufficient to block the apoptotic response mediated by the endogenous protein Bak [60, 61].

The ability of crmA and/or p35 to block apoptosis in distantly related organisms suggests a conserved role for certain viral proteins which target protease active sites. Likewise, BHRF1 and E1B 19 kDa appear to have selected the Bcl-2 family members to facilitate a viral advantage over apoptotic mechanisms in cells. The convergence of known viral activity at these points suggest that default apoptotic control in the multicellular organisms relies heavily on these pathways.

Links between Bcl-2 family oncoproteins and proteases in apoptosis

It has been shown that only the co-expression of Bcl-2 and Bag-1 (the Bcl-2-binding protein) completely inhibits Fas-mediated apoptosis [62]. Bag-1 contains a sequence domain which has similarity to ubiquitin and several ubiquitin-like proteins. Previous studies using a ubiquitin antisense approach have documented a requirement for ubiquitin during apoptosis induced by γ radiation in thymocytes [63]. It is possible that a ubiquitinated Bag-1 may play a role by bringing Bcl-2, or other Bcl-2-binding proteins, into contact with a protease or protease-containing protein complex (proteasomes) that participates in cell death. However, at this time the fate of Bag-1 is unknown. It is interesting to note that purified recombinant ICE specifically cleaves Bcl-x, but not Bcl-2 or Bax (J. M. Hardwick, pers. commun.). Recently, a novel cellular protein, Bik, has been shown to interact with the cellular survival-promoting proteins, Bcl-2 and Bcl-x_L, as well as with viral survival proteins EBV-BHRF1 and E1B 19 kDa [64]. Co-expression of these proteins suppressed the death-promoting activity of Bik, suggesting that Bik may be a common target for the cellular and viral survival-promoting proteins of the Bcl-2 family. These data provide evidence for a more direct interaction between effectors of cell death (proteases) and cellular survival factors (Bcl-2/Bcl-2-interacting proteins), placing turnpikes in the path of ICE-like protease(s) during the execution step of apoptosis mediated by diverse stimuli.

Substrates for apoptotic proteases

Several proteins have been suggested as potential targets for apoptotic proteases. Thus, it has been shown [23] that an enzyme with properties like ICE, but not ICE itself, is responsible for the specific proteolytic breakdown of poly(ADP-ribose) polymerase (PARP). This enzyme was purified from cells which spontaneously underwent apoptosis [65] and from cells treated with TNF [14] or with Fas [66, 67]. In each case, the isolated enzyme had the properties of ICE but, unlike ICE, was able to cleave PARP quite soon after induction. It is known that PARP is involved in DNA repair and genome integrity, an activity seen predominantly in response to treatment with DNA-damaging agents. It has also been proposed that PARP may negatively regulate the activity of Ca^{2+}/Mg^{2+}-dependent endonuclease(s), which has been implicated in the internucleosomal chromatin cleavage during apoptosis [68]. The loss of normal PARP function may render this endonuclease(s) active in dying cells. However, at least three observations make it difficult to consider cleavage of PARP as an important early event in the apoptotic process. First, PARP activity is linked to DNA damage which may lead to cleavage, often a relatively late event in apoptosis. Second, currently there are no known ICE-like protease(s) which have a nuclear location. PARP is a nuclear enzyme, and the protease which cleaves PARP should be present simultaneously in the nucleus. Finally, mice lacking the PARP gene are capable of efficient DNA repair [69].

It is interesting that another protein which plays a significant role in DNA strand-break repair, DNA-dependent protein kinase (DNA-PK), is also cleaved in apoptotic cells [70]. Although it is still unclear why PARP and DNA-PK are cleaved by apoptotic protease(s), one may speculate that it is important for the termination of DNA repair as a later event in the apoptotic cascade [70].

Another protein rapidly cleaved in apoptotic cells is the 70-kDa component of the U1 small ribonucleoprotein (U1-70 kDa) [71, 72]. U1-70 kDa cleavage activity contained in Fas- and TNF-induced cytosols was blocked by native crmA, but not by the crmA mutant which is also incapable of inhibiting ICE. Moreover, the sensitivity of U1-70 kDa cleavage to a panel of protease inhibitors was not identical to that observed for PARP cleavage in apoptotic cells. It was shown that YVAD

did not prevent the cleavage of U1-70 kDa [71], and when purified ICE was added to the lysates from control cells, U1-70 kDa was stable. It was suggested [71] that a loss of this protein may play a role in regulating mRNA splicing during apoptosis. U-RNP proteins have been characterized from the matrix attachment regions (MAR) [73], and may serve a dual function in the nucleus. They act first as an important component of hnRNP particles for mRNA splicing and second as an element of the higher-order structural organization of chromatin.

A second potential proteolytic target in the MAR, playing an important role in the binding of chromatin, is nuclear lamin. Indeed, lamin proteolysis was observed in many experimental systems [5, 44, 45, 74, 75]. Studies using a panel of protease inhibitors have provided evidence that the lamin and PARP proteases have distinct enzymatic activities [74]. The lamin protease appears to play an important role; when its activity is inhibited, the pathway of morphological changes is blocked at the stage of chromatin condensation [44, 45, 74]. As was discussed above, the adenovirus E1B 19 kDa protein confers cellular resistance to apoptosis. Recently, it was observed that E1B 19 kDa protein colocalizes with the nuclear lamina in different types of cells [61, 76]. The significance of this interaction is unknown but suggests that E1B 19 kDa may participate in the regulation of MAR function and possibly inhibits lamin cleavage.

A homology search of proteins containing cleavage sites next to P_1 asparagine has revealed that at least one other nuclear protein, Rel B, could be cleaved by an ICE-like protease. Rel B is a member of the NF-kB/Rel family of transcription factors. We have found (Zhivotovsky et al., unpublished observations) that in Fas-treated cells cleavage of Rel B occurred relatively quickly and preceded other biochemical and morphological nuclear changes. In contrast, Rel A, another member of this family, which does not contain the potential cleavage site, was stable during the experiment. The biological significance of Rel B cleavage is currently unknown.

Thus DNA-PK, PARP, U1-70 kDa, lamins and Rel B, as well as several other nuclear proteins (NuMA, retinoblastoma protein, topoisomerase I and II, and histone H1) which contribute to the maintenance of structural and functional integrity in the nucleus, all represent specific targets for apoptotic proteases including the ICE-like protease(s) [5, 43, 70, 71, 77–79]. The loss of these proteins will eventually result in irreversible changes to genome access and could block virus propagation. The specific cleavage of these proteins causing the disruption of nuclear integrity and homeostasis, regardless of time, seems to be a consequence of apoptosis rather than part of a signalling pathway.

Recently, it was demonstrated that cleavage of α-fodrin accompanied apoptosis induced by different stimuli [41, 80]. Data from our laboratory reveal that α-fodrin cleavage is related to membrane blebbing and is an important event in Fas-mediated apoptosis in Jurkat cells [41]. Since calpain activity is thought to be responsible for α-fodrin cleavage and has been observed during certain instances of apoptosis (as above), it seemed reasonable to test calpain inhibitors as well as inhibitors of other proteases in this system. Calpain inhibitors I and II did not protect against α-fodrin cleavage, membrane blebbing or chromatin fragmentation in this experimental system [41]. In contrast, all these effects of Fas-mediated apoptosis were prevented by VADcmk, which again points to a critical role of ICE-like protease in the effector consequences of apoptosis.

Recent work has identified a number of other cytosolic proteins that are selectively cleaved during apoptosis, including PLA2; PKCδ; PITSLRE protein kinase; sterol regulatory element-binding proteins; cytoskeletal proteins 11 (actin and vimentin); a cytoskeleton-associated protein (Gas2) and GDP dissociation inhibitor (D4-GDI) [81–87]. Precisely how the cleavage of these proteins is involved in apoptosis remains unknown, although it has been suggested that disruption/cleavage of cytoskeletal proteins might promote cell shrinkage, membrane blebbing and decrease intracellular contact. The cleavage of PLA2, PKCδ and D4-GDI may deregulate their respective enzymatic activities, thereby changing signalling pathways and altering any control of cell survival. Schematic representation of the possible involvement of proteases in apoptosis and their potential targets is presented in figure 1.

Are the apoptotic proteases cytoplasmic enzymes?

There is increasing evidence that the biochemical machinery mediating the apoptotic process is constitutively expressed and subject to activation by various signals. Recent studies with enucleated cells (cytoplasts) suggest that the apoptotic process can be initiated and proceed in the absence of the nucleus [88]. Since there may be a 'cytoplasmic regulator' of this process, one might speculate that the nature of this regulator could be a protease or a complex of various proteases. Using cell-free systems, several groups have attempted to analyse protease(s) and determine the sequence of events leading to and predicting target protein cleavage [89–91]. Cell lysates prepared after treatment with various agents have been shown to induce chromatin fragmen'-tation as well as morphological changes in isolated native nuclei. Pretreatment of the cells with inhibitors of serine proteases (TPCK and DCI) or ICE-like proteases (VAD or YVAD) blocked the induction of this 'cytosolic activity'. The exogenous addition of purified Bcl-2 led only to partial inhibition of lysate activity isolated from Fas-treated cells. Morphological and biochemical changes in the lysate-subjected nuclei were not equally

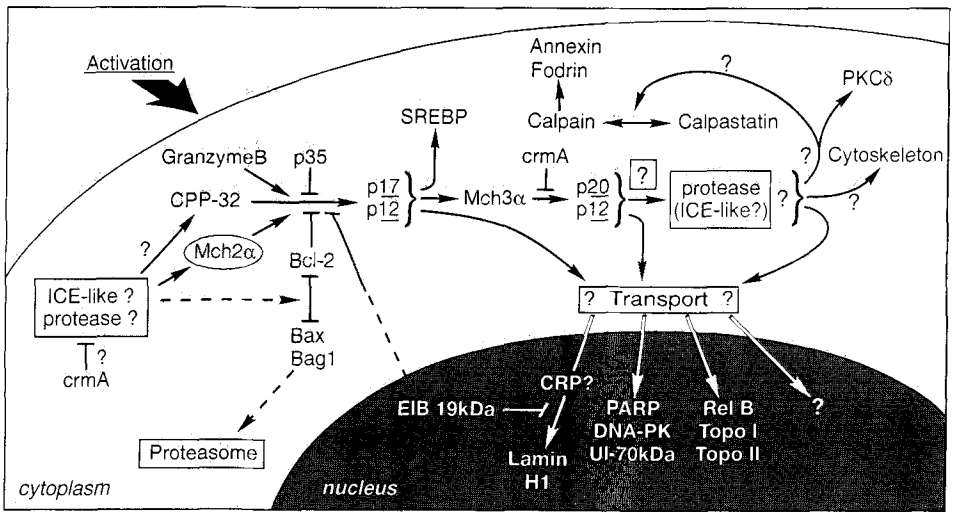

Figure 1. Schematic representation of the possible involvement of proteases in apoptosis and their potential targets.

blocked by different inhibitors, suggesting the involvement of distinct proteases and substrate susceptibility during apoptosis. It is important to note that when the tetrapeptide inhibitor YVAD, or VAD, were added to the cell lysates, they had little effect on subsequent nuclear DNA degradation [89, 90]. These results suggest that inhibitors may block the activation of cytoplasmic effector ICE-like enzymes, but that once activated, these or other enzymes become insensitive to the inhibitors.

Are CPP32/apopain or Mch3α/ICE-LAP3/CMH1 Fas- and TNF-activated proteases?

Strong candidates for the role of FasR-activated ICE-like proteases have been cloned [11, 13, 18, 19]. Human CPP32 and apopain have been identified and are the same 32-kDa protease zymogen (despite a probable sequence polymorphism). CPP32 mRNA and protein appear to be constitutively produced in almost all of the cell types and tissues tested [11, 65]. Within 15 min of FasR crosslinking, the specific cleavage of CPP32 results in the formation of the p17 and p12 fragments of the active enzyme apopain. Heterodimers of p17/p12 most likely pair to form tetramers, which can cleave the substrate analogue DEVD-AMC in vitro and PARP in vitro and in vivo. Recombinant CPP32 does not induce PARP cleavage in isolated nuclei, but requires the addition of native cytosol which apparently provides this activity. Both PARP cleavage and the proapoptotic effects of this protease are inhibited by nanomolar concentrations of Ac-DEVD-CHO, while an inhibitor of ICE (Ac-YVAD-CHO) requires micromolar concentrations to effect a comparable inhibition.

Experiments using a reconstituted in vitro system containing cytosolic extracts from Jurkat cells undergoing Fas-induced apoptosis were undertaken to characterize a protease that promotes apoptotic changes in isolated nuclei [66, 67]. This system identified a proteolytic activity which was inhibited by several serine protease inhibitors, iodoacetamide and VAD-cmk, while E64 was ineffective. This inhibitor profile is consistent with the protease being a member of the ICE family. Further experiments revealed that this protease cleaves PARP with high efficiency and specificity. Analysis of the isolated protease revealed kinetic constants similar to those reported for apopain [65]. Furthermore, the protease was recognized by antibodies specific for CPP32/apopain but not by an anti-ICE antibody. These results suggest that CPP32/apopain is activated in Fas-mediated apoptosis and plays a prominent role in signalling apoptotic events in this experimental system.

Mch3α/ICE-LAP3/CMH1 has subsequently and independently been cloned by three groups [13, 18, 19]. This ICE-like zymogen is 35 kDa and is most closely related to CPP32 (53–57% homologous). The pentapeptide QACRG plus the His and Gly residues, which define the catalytic centre in ICE-like members, are conserved. Within 1 h of Fas or TNF treatment, full-length ICE-LAP3 undergoes a cleavage from p35 to a p20 fragment (probably p12 as well). This activation is inhibited by crmA but not by crmA mutant. Recombinant Mch3α was shown to rapidly cleave purified and isolated HeLa nuclei PARP, whereas CPP32 did not. In Sf9 cells, the co-expression of rCPP32 and rMch3α truncated clones demonstrated that there is a potential for functional interchange of p12 subunits resulting in the induction of apoptosis. Interestingly, Mch3α DNA sequences were recovered from a second cDNA clone, Mch3β, which represents an alternatively spliced mRNA that might possibly give rise to an inactive/inhibitory p20 protein. Although Mch3α is closely related to CPP32, the latter can efficiently cleave proMch3α, but not the converse. Moreover, active CPP32 is present within 15 min of Fas

activation and appears to precede Mch3α fragmentation by at least 30 min. This suggests that Mch3α activation in vivo may depend in part upon the presence of active CPP32/apopain. Whether the native isolate of apopain is likewise capable of cleaving Mch3α is currently unknown.

Receptor-mediated activation of apoptosis via FADD/MORT1-associated ICE-like proteases may soon be examined in detail. The MACH/FLICE protein, which has most recently been reported [92, 93], represents an unusual class of cysteine protease(s) which has ICE-like properties located in C-terminal and death domains homologues in N-terminal regions. The FLICE protein is homologous to one of the CAP proteins (cytotoxicity-dependent Fas/APO-1-associated proteins) previously reported by Kischkel et al. [94], and in the current paper by Muzio et al. [92] is shown to be associated with CD95-mediated DISC formation upon agonist antibody activation. The activation of FLICE results in the production of p20 and p10 subunits and the ability to cleave PARP. Likewise, MACH has been shown in vitro to be capable of cleaving the DEVD-AMC substrate reminiscent of the PARP cleavage site [93]. Interestingly, MACH mRNAs appear to contain alternative splice forms. One can speculate that there is potential for heterogeneity at the protein level which may occur in various cell types. Combined, these data show that MACH/FLICE, CPP32/apopain and Mch3α/ICE-LAP3/CMH1 are proteases involved in receptor-mediated events during apoptosis.

Although it has not been defined as being activated by the Fas or TNF receptors, a hamster homologue of the human ICE-like Mch2α has recently been characterized as having CPP32-activating protease (CAP) capability [95]. Purified CAP can cleave crmA and CPP32 but is relatively less efficient at cleaving SREBP than apopain activity. The human Mch2α is proficient at cleaving PARP and DEVD-AMC, thereby having an activity similar to CPP32. It is tempting to speculate that when activated the Mch2α homologue may be involved in cleavage of certain endogenous serpinlike inhibitors of apoptosis as well as the coincident cleavage of CPP32. Indirectly, this suggests that within cells a possibility exists for the sequential activation of CPP32 by Mch2α which might in turn activate Mch3α. How the activation of such a pathway is linked to receptor-mediated events or to other forms of apoptosis induction will undoubtedly remain the goal of research in the coming years.

Specificity of substrate cleavage and the relationships amongst the ICE-like proteases

ICE is autocatalytic, which places proICE as the ultimate substrate in this series. ICE is highly specific for proIL-1β, and there are some indications that ICE can cleave proYAMA and proNedd2(murine), albeit with much lower specificity (table 1) [10, 14]. Ich2, TX and $ICE_{rel}II$ are also autocatalytic and can cleave proICE; however, none of these can cleave proIL-1β [15–17]. There are no indications that two other closely related proteases, $ICE_{rel}III$ and Ich1/muNedd2, cleave proICE or proIL-1β. Although PARP can be cleaved by all of the above named proteases, this activity requires very high enzyme-to-substrate ratios relative to the native enzyme activity. Thus, human ICE, Ich2, TX, $ICE_{rel}II$, $ICE_{rel}III$ and Ich1/muNedd2 have very restricted cellular targets, which makes it difficult to confirm their involvement in apoptosis at the moment.

Table 1. ICE-like proteases and possible substrates/zymogen targets.

Enzyme	Substrate	Zymogen
CPP32/Apopain/Yama	PARP, DNA-PK, U1-70 kDa, SREBP	Mch3α
Mch3α/ICE-LAP3/CMH1	PARP	?
Mch2α	?	CPP32
muNedd2	PARP*	proICE
huIch-1	PARP*	?
$huICE_{rel}II$	PARP*	proICE
$huICE_{rel}III$	?	?
huICE	proIl-1β	proICE
Ich-2/TX	proIl-1β	proICE/Ich-2

*This substrate can be cleaved with 100-fold excess of the CPP32:substrate ratio. '?' denotes currency unknown.

A second group of proteases cleaves neither pro-ICE nor proIL-1β. Instead, this group cleaves protein substrates which are apparently cellular targets during apoptosis. CPP32/apopain/YAMA, Mch2α and Mch3α/ICE-LAP3/CMH1 cleave PARP. CPP32 can also cleave SREBP [84], DNA-PK and U1-70 kDa [96]. There is no evidence that CPP32 is autocatalytic. Pro-Mch3α appears to be a cleavage target for activated CPP32 and activated Mch3a itself, but CPP32 is not a substrate for Mch3a [13]. As previously mentioned, Mch2α can cleave CPP32, but the mechanism for activation of this initial cascade is still unclear.

Conclusion

The ultimate goals of apoptosis are the orderly destruction of transferable genetic material and the noninflammatory removal of cellular remnants. A critical question which faces researchers is how to explain the apparent segregation of cytoplasmic effector enzymes from their nuclear targets. The ability of activated zymogen(s) to vertically transfer and execute a functional activity within the nuclear milieu must be confirmed in order to coalesce the cascade of apoptotic signals with the fulfilment of both apoptotic goals. One can speculate that a cascade of different proteases is involved. The activation of one could lead to auto- or allo-substrate recognition within a protease class, resulting in successive

and/or parallel steps along a pathway. For example, CPP32 appears necessary but not sufficient to induce nuclear changes related to apoptosis. For activation, CPP32 requires an endogenous signal which leads to its proteolytic and perhaps semi-autocatalytic processing. Candidates for this during receptor-mediated activation might possibly come from the ICE-like family. The fact that granzyme B cleaves CPP32 [27] in vitro probably represents the immune system's efficient way of bypassing receptor-mediated events to activate a universal default pathway directly. An endogenous protein effecting the same cleavage as granzyme B, thereby activating CPP32, will likely be a receptor-linked initiator in vivo. Potential candidates for such initiators include Mch2α, and the interplay between FLICE/MACH-like and CPP32-like proteases which is still to be defined [13, 95]. Currently an activated CPP32 represents the proteolytic 'point of no return', the first known effector in this cascade. Apopain's ability to cleave different DNA repair/processing proteins clearly suggests a specific role for CPP32-like proteases during the disruption of cellular homeostasis [96]. If each of the CPP32-like enzymes evokes, with some redundancy, similar disruptions in cellular homeostasis, then clearer links between induction and effector function may be appreciated. Within active CPP32/apopain a cytoplasmic Mch3α-convertase activity also occurs. Subsequent convertase activity harboured within Mch3a is currently unknown and represents an area of great interest.

To disregard the involvement of other ICE-like members in the FLICE/MACH, Mch2α, CPP32 and Mch3α protease interactions suggested above may very well be oversimplistic and premature. The potential exists for intratetrameric assembly of subunits within this protein family. In different cells and tissues zymogen interactions with subunit protein products of alternatively spliced mRNA could change enzyme activation or substrate specificity and lead to divergent proteolytic pathways. A loss of enzyme activity in this way may result in the cessation of receptor-mediated apoptotic events seen in some cell types committed to particular functions. Possibly, changes in protease specificity might also lead to ion flux perturbations, disruption of endogenous protease inhibitors, and changes in redox potential and ubiquitin targeting processes, any of which could unleash a broad range of cytosolic and nuclear proteolytic pathways. Certainly, the specific proximal enzyme cascades which commit cells to apoptosis, and the more distal events resulting in massive proteolysis, will become more distinct as this research continues.

Note added in proof: Meh4 has recently been shown to cleave and activate several members of the CPP-32-like proteases in vitro [Fernandes-Alnemri T., et al. (1996) PNAS **93:** 7464–7469].

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