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Proteases in Fas-Mediated Apoptosis

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Abstract Involvement of a unique family of cysteine proteases in the multistep apoptotic process has been documented. Cloning of several mammalian genes identifies some components of this cellular response. However, it is currently unclear which protease plays a role as a signal and/or effector of apoptosis. We summarize contributions to the data concerning proteases in Fas-mediated apoptosis. J. Cell. Biochem. 64:43–49. © 1997 Wiley-Liss, Inc.

Key words: apoptosis; Fas; proteases; substrates

Interest in the role of intracellular proteases during apoptosis was markedly stimulated by the observation that both the *Caenorhabolitis elegans* cell death gene, *ced-3*, and its mammalian homologue, interleukin-1β-converting enzyme (ICE), contain a conserved pentapeptide domain at the active site (Yuan et al., 1993). Since this observation, additional members of ICE gene family, with cysteine protease properties, have been cloned (Ich-1/Nedd-2, Mch-2, Mch-3, and CPP32/apopain/YAMA). Overexpression of any one of these leads to apoptotic cell death in various systems. Although it appears that ICE family members play a central role in promoting apoptotic cell death, some evidence has been advanced that other proteases are also involved. This past year, several articles have described the involvement of different proteases in experimental models of apoptosis. Here we attempt to summarize the current thinking concerning these proteases and their involvement in Fas-mediated apoptosis.

FAS-MEDIATED APOPTOSIS: TRIGGERING (SIGNALING MODE)

Cross linking of Fas/APO-1 (CD95), a member of TNF receptor (TNFR) family, with antibody or its natural ligand (FasL), transduces an extremely rapid apoptotic signal in a variety of cell types. This transduction is mediated through cytoplasmic domains of FasR (Fas receptor) and may require the binding of several candidate cytoplasmic proteins, MORT1/FADD, TRADD, and RIP. Each of these contain homologous protein motifs, death domains [Itoh et al., 1993; Tartaglia et al., 1993], which are similiar to the cytoplasmic domains required to transduce TNFR-1 mediated signals [Boldin et al., 1995; Chinnaiyan et al., 1995; Stanger et al., 1995]. Overexpression of TRADD promotes cell death, which is consistent with the death domain binding during transient overexpression of FADD or RIP, themselves capable of bypassing receptor mediated events and lead directly to apoptosis. Varfolomeev et al. [1996] investigated the interaction among MORT1/FADD, RIP, and the FasR and proposed that it is distinct from TRADD, RIP, and TNFR-1 interactions. Kischkel et al. [1995] showed a cytotoxicity-dependent requirement for a set of Fas/APO-1-associated proteins (CAP1-4) that interact with oligomerized but not monomeric FasR. CAP1 and CAP2 were shown to be identical to MORT1/FADD; however, the origin and role of CAP3 and CAP4 is unclear. A hypothesis put forth by Kischkel et al. [1995] is that CAP4 maybe proteolytically processed RIP. Further investigations will be required to clarify the death-domain and CAP protein involvement in the Fas-induction pathway.

FasR cross linking results in an increase of cellular tyrosine phosphorylation events [Eischen et al., 1994]. FAP-1, a tyrosine phos-

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phatase, has been shown to interact with the cytosolic domain of Fas [Sato et al., 1995]. The overexpression of FAP-1 partially abolished Fas-induced apoptosis. Therefore, FAP-1 and protein tyrosine-kinase(s)-mediated events may regulate Fas signal transduction. This implies the involvement of a protein tyrosine phosphatase/kinase system as part of the signaling mode during Fas-mediated cytotoxicity.

Other signaling events have been suggested for Fas-mediated apoptosis. Preliminary work shows that elevations in the cytosolic Ca^{2+} level [Oshimi and Miyazaki, 1995], production of ceramide [Gulbins et al., 1995], and redistribution of TIAR, a TIA-1-related RNA-binding protein [Taupin et al., 1995] are involved. No direct evidence links these signals to either activation (signaling) of the protease(s) or cleavage (effector) of known protein substrates.

Recent work in our laboratory has shown that the serine protease inhibitors prevent Fas-induced apoptosis in Jurkat cells, suggesting that distinct proteolytic enzymes play a central role in this type of cell death [Weis et al., 1995]. Furthermore, in Fas-mediated apoptosis, some isoforms of PITSLRE protein kinase may be targeted by apoptotic proteases and blocked by inhibitors of serine proteases [Lahti et al., 1995]. Although both studies point to serine protease activation as early events in Fas-activated cells, neither can specify which particular proteases are involved in this form of apoptosis.

IS ICE INVOLVED IN FAS-MEDIATED APOPTOSIS?

By using the tetrapeptide YVAD, two groups independently demonstrated a dose-dependent inhibition of the anti-Fas initiated apoptotic process [Enari et al., 1995b; Los et al., 1995]. Fas rapidly stimulated the proteolytic cleavage of the test ICE-like substrate. Overexpression of ICE strongly potentiated Fas-mediated cell death. Transient expression of antisense ICE resulted in an inhibition of Fas-induced apoptosis by 50% [Los et al., 1995]. However, it is necessary to point out that inhibitors and substrates used in these studies could delineate the activity of ICE and of ICE-like proteases [Lazebnik et al., 1994]. This idea was supported by the finding that ICE-deficient mice exhibit few defects in apoptotic cell death and proceed through development normally in contrast with *ced-3* mutants of *C. elegans* [Kuida et al., 1995; Li et al., 1995]. Moreover, cell lines with undetectable level of ICE were sensitive to Fas treatment, and Fas-mediated apoptosis in these cells was not inhibited by the ICE inhibitors [Hasegawa et al., 1996]. In addition, secretion of IL-1β occurs from monocytes, mediated by active ICE at the plasma membrane, with no demonstrable evidence of apoptosis. Singer et al. [1995] concluded that IL-1β secretion does not involve apoptotic machinery, and apoptosis itself is probably driven by other distinct ICE-like proteases under tight regulatory control.

ROLE OF VIRAL PROTEINS

ICE appears to be inhibited by crmA, a pox virus protein that apparently mediates the virus block of host-cell suicide. CrmA, a member of the serpin family, inhibits ICE by forming an active site-directed complex. Therefore, it was hypothesized that crmA might have antiapoptotic activity in addition to its ability to inhibit processing of IL-1β. Transfection of crmA into different cell types conferred resistance to apoptotic activity of anti-Fas antibody [Enari et al., 1995b; Los et al., 1995; Tewari and Dixit, 1995]. This inhibition by crmA was "transfection" dose dependent [Enari et al., 1995b]. In this case, the stochiometry of crmA and effected cellular proteins cannot be determined. The dose-dependent inhibition of substrate cleavage shows that crmA is a 10,000-fold more effective inhibitor of ICE mediated IL-1β cleavage than is CPP32 cleavage of poly(ADP-ribose)polymerase (PARP) [Nicholson et al., 1995]. It is still unclear which steps of apoptosis are most susceptible to the crmA inhibitory effect.

CrmA is not the only viral protein shown to block apoptosis. Evidence for the baculovirus protein p35 inhibition of ICE-like protease activity was shown in an enzymatic assay [Bump et al., 1995; Beidler et al., 1995; Xue and Horwitz, 1995]. Inhibition of enzymatic activity correlated with the formation of a stable protease–p35 complex [Bump et al., 1995]. As with crmA, the coexpression of p35 gene product inhibits virally induced apoptosis, programmed cell death in *C. elegans* and *Drosophila*, neuronal cells death, and TNF and Fas-mediated apoptosis. However, unlike crmA, p35 has no effect on granzyme B [Bump et al., 1995]. The ability of crmA and p35 to block apoptosis in distantly related organisms suggests a conserved role for ICE-like proteases and a negative regulatory role for these viral proteins at sites of convergence in the signaling mode.

Adenovirus E1B 19-kDa protein confers cellular resistance to cytotoxicity from TNF and

anti-Fas antibody [Hashimoto et al., 1991]. The molecular basis of this inhibition was poorly understood; however, Chiou et al. [1994] showed a functional similarity between Bcl-2 and the E1B 19-kDa protein that suggests a similar mechanism of action for these death-defending proteins. E1B 19 kDa alone protects cells from apoptosis, which suggests that E1B 19 kDa may target certain cellular proteins. Farrow et al. [1995] identified three proteins that bind E1B 19 kDa. One of these is Bak, a member of the Bcl-2 family. Overexpression of Bak accelerates several types of apoptosis and blocks the protective effect of E1B 19 kDa. Conversely, increased levels of E1B 19 kDa is sufficient to block the apoptotic response mediated by Bak. Whether this interaction is common for all types of apoptosis or restricted to apoptosis mediated by Neuronal growth factor withdrawal [Farrow et al., 1995] remains to be determined.

ROLE OF ONCOPROTEINS

Overexpression of Bcl-2 oncoprotein suppresses apoptosis by different inducers [for review, see Korsmeyer, 1992]. Purified recombinant Bcl-2 and Bcl-2-containing cell lysate inhibit in vitro apoptosis induced by Fas-activated cell lysates [Enari et al., 1995a; Martin et al., 1995b]. However, inhibition of Fas-induced cell death by Bcl-2 overexpression was demonstrated to be only partially effective [Itoh et al., 1993]. Coexpression of Bcl-2 and Bag-1 (the Bcl-2 binding protein) completely inhibits Fas-mediated apoptosis [Takayama et al., 1995]. Whether Bag-1 enhances the inhibitory activity of Bcl-2 in vitro is still unknown. It is interesting to note that purified recombinant ICE specifically cleaves Bcl-x, but not Bcl-2 or Bax (J.M. Hardwick, personal communication). The fate of Bag-1 is unknown. These data provide evidence for a direct interaction between effectors of cell death and survival factors by placing Bcl-2 in the path of ICE-like protease(s) during the execution of Fas-mediated apoptosis.

Bcl-2 is not the only oncoprotein implicated in the regulation (protection) of apoptosis. Other oncoproteins, such as c-ErbB-2, were able to induce the resistance to TNF treatment [Hudziak et al., 1988]. A recent publication by McGahon et al. [1995] presented evidence for the negative regulation of the Fas-mediated apoptosis by the Abl oncoprotein. The viral homologue of the c-abl gene product is a tyrosine kinase. Because Abl was also found to block apoptosis mediated by ceramide, which may be a downstream effector of the apoptotic pathway induced by Fas, McGahon et al. [1995] believed that Abl manifests its effects after the early Fas-initiated signaling events. However, the ceramide pathway involved activation of Ha-Ras. Ras by itself is involved in the signaling events leading to v-abl-mediated cell transformation [Stacey et al., 1991]. Whether Ras activation by Abl is an important step in the prevention of Fas-induced apoptosis is still unclear.

IS FAS-MEDIATED PROTEASE A CYTOPLASMIC ENZYME?

There is increasing evidence that the biochemical machinery involved in mediating the apoptotic process is expressed constitutively and is subject to activation by various signals. In fact, recent studies with enucleated cells (cytoplasts) have suggested that the apoptotic process can be initiated and proceed in the absence of the nucleus and that there may be a "cytoplasmic regulator" of the process. Because the nature of this regulator is yet unknown, it could be a protease or a complex of various proteases. By using a cell-free system, several groups have attempted to analyze protease(s) and determine the sequence of events predicting the target proteins [Chow et al., 1995; Enari et al., 1995a; Martin et al., 1995b]. Cell lysates prepared from Fas-treated cells induced chromatin fragmentation and morphological changes in isolated nuclei. Pretreatment with inhibitors of serine proteases (TPCK and DCI) or ICE-like proteases (VAD or YVAD) blocked the induction of "cytosolic activity." Addition of purified Bcl-2, however, leads to only partial inhibition of lysate activity. Morphological and biochemical changes in isolated nuclei in the presence of cytoplasmic extract were not equally blocked by different inhibitors, suggesting the involvement of different proteases during Fas-mediated apoptosis. When the tetrapeptide inhibitor was added to activated cell lysates, it had little effect on the progression of chromatin cleavage [Chow et al., 1995; Enari et al., 1995a]. This result suggests that these inhibitors can block the activation of cytoplasmic signaling ICE-like enzymes; however, once activated, the components of this pathway become insensitive to certain inhibitors.

SUBSTATES FOR FAS-MEDIATED PROTEASES (EFFECTOR MODE)

Several proteins have been suggested as potential targets for protease activity. Lazebnik et

al. [1994] showed that an enzyme with the properties like ICE, but not ICE, is responsible for the specific proteolytic breakdown of PARP. Occurrence of Fas-mediated protein cleavage is presented in Figure 1. This enzyme was purified from cells that spontaneously underwent apoptosis [Nicholson et al., 1995] or cells treated with TNF [Tewari et al., 1995b] or with Fas [Schlegel et al., 1995, 1996]. In each case, the isolated enzyme had the properties of ICE but was able to cleave PARP quite soon after induction. PARP is involved in DNA repair and genome integrity, an activity seen predominantly in response to treatment with DNA-damaging agents. PARP also negatively regulates the activity of Ca^{2+}/Mg^{2+}-dependent endonuclease, which was implicated in the internucleosomal chromatin cleavage [Nelipovich et al., 1988]. The loss of normal PARP function may render this nuclease active in dying cells. However, at least two facts make it difficult to consider cleavage of PARP as an important early event in the apoptotic process. First, the activity of PARP is linked to DNA damage or cleavage, which is a relatively late event in apoptosis. Second, PARP is a nuclear enzyme, and the protease that cleaves PARP should have a nuclear location. However, there are no ICE-like proteases that are to date known to be nuclear. The biological meaning of PARP cleavage as a target of protease activity is unknown.

Another proteolytic substrate rapidly cleaved after Fas and TNF treatment is the 70-kDa component of the U1 small ribonucleoprotein (U1-70 kDa) [Tewari et al., 1995a]. This cleavage was potentially blocked by native CrmA but not by its mutated form, which is also incapable of inhibiting of ICE. Moreover, the sensitivity of U1-70 kDa to protease inhibitors was not completely identical to that observed for PARP cleavage in apoptotic cells. Thus, YVAD did not prevent the cleavage of U1-70 kDa [Casciola-Rosen et al., 1994]. When purified ICE was added to lysates of control or apoptotic cells, U1-70 kDa was stable. A loss of this protein may play a role in regulating RNA splicing during apoptosis [Casciola-Rosen et al., 1994]. U-RNP proteins have been characterized from the matrix attachment regions (MAR) [von Kries et al., 1994]. U-RNP may serve a dual function: first, as a component of hnRNP particles important for splicing and, second, as an element in the higher order organization of chromatin. A second potential proteolytic target in the MAR playing an important role in the binding of chromatin is nuclear lamin.

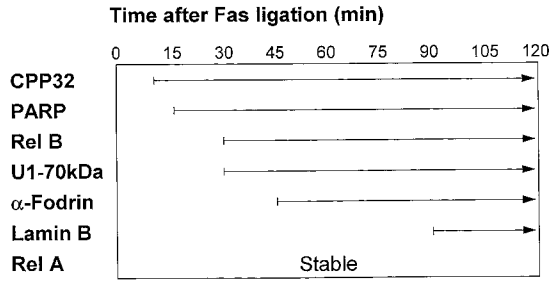

Fig. 1. Occurrence of substrate cleavage in Fas-treated cells.

Lamin proteolysis was observed in many experimental systems [Kaufmann 1989; Lazebnik et al., 1995, Neamati et al., 1995; Zhivotovsky et al., 1995], including Fas-mediated apoptosis [Zhivotovsky et al., submitted]. In Fas-treated cells, the cleavage of PARP and lamin was detected at 15 min and 1 h, respectively. Studies using a panel of proteinase inhibitors have provided evidence that the lamin and PARP proteinases have distinct enzymatic activities [Lazebnik et al., 1995]. The lamin proteinase appears to play an important role; when its activity is inhibited, the pathway of morphological changes is blocked at the stage of chromatin condensation [Lazebnik et al., 1995; Neamati et al., 1995; Zhivotovsky et al., 1995]. As discussed under the Role of Viral Proteins section, the adenovirus E1B 19-kDa protein confers cellular resistance to Fas-induced cytotoxicity. E1B 19 kDa protein has been observed to colocalize with the nuclear lamina in different types of cells [White and Cipriani, 1989; Farrow et al., 1995]. The significance of this interaction is unknown but suggests that E1B 19 kDa may participate in the regulation of MAR function and possibly inhibit 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., submitted] that in Fas-treated cells Rel B underwent proteolysis relatively quickly, preceding biochemical and morphological changes in nuclei. In contrast, another member of this family, Rel A, which does not contain the potential cleavage site, was stable during the experiment. The biological meaning of Rel B cleavage is currently unknown.

Thus, these resident nuclear proteins (PARP, U1-70 kDa, lamins, and Rel B) contribute to

maintaining structural and functional integrity of the nucleus and represent specific targets for ICE-like protease(s). Proteolysis of these proteins will eventually result in an irreversible loss of genome access and could block virus propagation. Cleavage of these proteins, regardless of time, seems to be an effector consequence of apoptosis rather than part of induction.

Recently, it was demonstrated that cleavage of α-fodrin accompanied apoptosis, induced by different stimuli including Fas [Martin et al., 1995a; Vanags et al., submitted]. α-Fodrin cleavage during apoptosis may be related to the process of membrane blebbing seen during this process. Data from our laboratory show that α-fodrin cleavage accompanies membrane blebbing and a significant increase in the exposure of phosphatidylserine (PS) on the surface of Fas-treated Jurkat cells [Vanags et al., submitted]. Because calpain activity has been observed during certain instances of apoptosis (Squier et al., 1994), it seemed reasonable to test calpain inhibitors and inhibitors of other proteinases in this system. Calpain inhibitors I and II did not protect against either α-fodrin cleavage, PS exposure, blebbing, or chromatin fragmentation [Vanags et al., submitted]. In contrast, all these steps of Fas-mediated apoptosis were significantly protected by VADcmk, which again points to the critical role of ICE-like protease in the effector consequence of apoptosis in this system. The precise role of calpain in Fas-mediated apoptosis remains to be elucidated.

IS CPP32 A FAS-MEDIATED PROTEASE?

Experiments using a reconstituted in vitro system containing cytosolic extracts from cells undergoing Fas-induced apoptosis were undertaken not only to characterize but also to purify a protease that promotes apoptotic changes in isolated nuclei [Schlegel et al., 1995, 1996]. This proteolytic activity was inhibited by two serine protease inhibitors, iodoacetamide and VAD-cmk, whereas E64 was not inhibitory. This inhibitor profile is consistent with the protease being a member of the ICE family. Recent experiments performed in our group show that this protease cleaves PARP with high efficiency and specificity. Both PARP proteolysis and the proapoptotic effects of the protease were inhibited by nanomolar concentrations of a selective inhibitor of CPP32/apopain (Ac-DEVD-CHO), whereas an inhibitor of ICE (Ac-YVAD-CHO) required micromolar concentrations for the inhibition of the isolated protease. Kinetic analysis of the isolated protease reveals constants similar to those reported for apopain [Nicholson et al., 1995]. The isolated protease was recognized by antibodies specific for CPP32/apopain but not by an anti-ICE antibody. These results provide strong evidence that CPP32/apopain is activated in Fas-mediated apoptosis and plays a key role in the signaling of apoptotic events in this experimental system. Schematic representation of the possible involvement of proteases in Fas-mediated apoptosis is presented in Figure 2.

Are proteases a component of signaling or effector modes in cells undergoing apoptosis? One can speculate that different proteases are involved in consecutive steps. Activation of one can lead to processing either molecules of the same or different proteases, thus leading to an activation of a cascade of proteases. For ex-

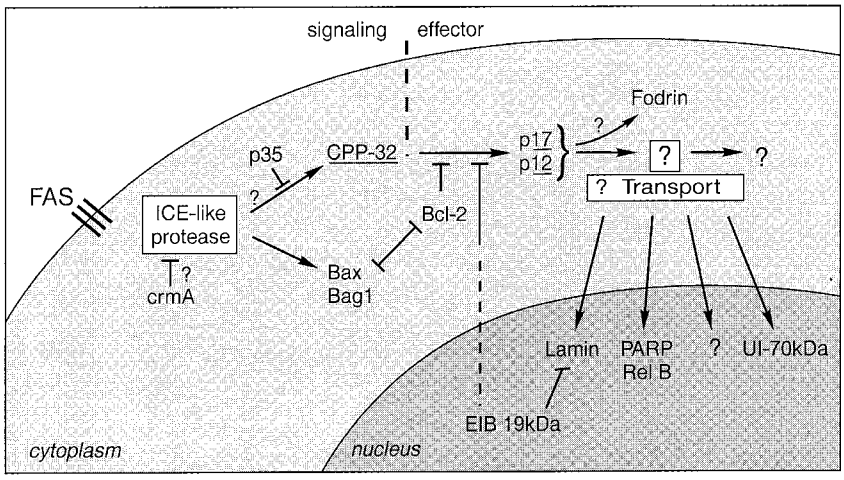

Fig. 2. Schematic representation of the possible involvement of proteases in Fas-mediated apoptosis.

ample, precursor CPP32 is proteolytically but not autocatalytically processed to form a holoenzyme composed of 17-kDa and 12-kDa subunits [Nicholson et al., 1995]. It is likely that an unknown enzyme cleaves CPP32 and initiates the proteolytic cascade, thus becoming the earliest step in induction of apoptosis. A possible candidate is a protein from the ICE family. In vitro granzyme B cleaves CPP32 [Darmon et al., 1995]. If an unknown protein from the ICE family effects the same cleavage as granzyme B, activating CPP32/apopain, it is likely that the former is an initiator in vivo. Currently, activated CPP32/apopain represents a proteolytic "point of no return," the first effector in this cascade.

NOTE ADDED IN PROOF

During the four months between the acceptance of this paper in May 1996 and the receipt of proofs in August 1996, two very important papers have contributed to understanding the links between FasR and ICE-like proteases (Boldin M et al., 1996, Cell, 85:803–815: Muzio M. et al., 1996, Cell, 85:817–827).

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