**FLICE Induced Apoptosis in a Cell-free System**

**CLEAVAGE OF CASPASE ZYMGENS**

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**Engagement of CD95 or tumor necrosis factor 1 receptor (TNFR-1) by ligand or agonist antibodies is capable of activating the cell death program, the effector arm of which is composed of mammalian interleukin-1β converting enzyme (ICE)-like cysteine proteases (designated caspases) that are related to the Caenorhabditis elegans death gene, CED-3. Caspases, unlike other mammalian cysteine proteases, cleave their substrates following aspartate residues. Furthermore, proteases belonging to this family exist aszymogens that in turn require cleavage at internal aspartate residues to generate the two-subunit active enzyme. As such, family members are capable of activating each other. Remarkably, both CD95 and TNFR-1 death receptors initiate apoptosis by recruiting a novel ICE/CED-3 family member, designated FLICE/MACH, to the receptor signaling complex. Therefore, FLICE/MACH represents the apical triggering protease in the cascade. Consistent with this, recombinant FLICE was found capable of proteolytically activating downstream caspases. Furthermore, CrmA, a pox virus-encoded serpin that inhibits Fas and tumor necrosis factor-induced cell death attenuates the ability of FLICE to activate downstream caspases.**

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**Apoptosis, or programmed cell death, is a cell deletion mechanism that is critical to metazoan survival (1, 2). The cell death machinery is conserved throughout evolution and is composed of several distinct parts including effectors, inhibitors, and activators (2, 3).**

**It is becoming apparent that mammalian cysteine proteases (designated caspases)**¹ related to the *Caenorhabditis elegans* cell death gene CED-3 represent the effector components of the apoptotic machinery. The first mammalian homolog of CED-3 to be identified was interleukin-1β converting enzyme (ICE) (4). Further studies, however, suggested that proteases related to ICE, rather than ICE itself, may play a more central role in the apoptotic mechanism. To date, 10 homologs of CED-3 and ICE (caspase-1) have been characterized and include Nedd-2/ICH1 (caspase-2) (5, 6), Yama/CPP32/apopain (caspase-3) (7–9), Tx/ICH2/ICE rel-II (caspase-4) (10–12), ICE rel-III, (caspase-5) (12), Mch2 (caspase-6) (13), ICE-LAP3/Mch3/CMH-1 (caspase-7) (14–16), ICE-LAP6 (caspase-9) (17), Mch4/Flice2 (caspase-10) (18), 2 Ich3 (caspase-11) (20), and FLICE/MACH (caspase-8) (21, 22). Ectopic expression of these ICE/CED-3 homologs in a variety of cells induces apoptosis. However, only Yama, LAP3, and Mch2 have been shown to be proteolytically activated by apoptotic stimuli including engagement of the CD95 and TNFR-1 receptors (14, 23, 24). Both these receptors utilize the adaptor molecule FADD as a conduit to relay death signals into the cells’ interior. Indeed, a discrete domain within FADD, designated the death effector domain, was found capable of engaging the cells death machinery and inducing apoptosis (25–27). The surprising revelation was the finding that the death effector domain of FADD bound to corresponding sequence motifs within the prodomain of FLICE/MACH and thereby recruited this putative death protease to the receptor signaling complex (21, 22). This suggested that FLICE was the apical triggering protease and should be competent to initiate proteolytic activation of downstream caspase family members resulting in apoptotic demise. The pox virus-encoded serpin cytokine response modifier A gene (CrmA) binds with differential affinity to the active forms of ICE and ICE-like proteases (8) and blocks cell death triggered by either death receptor (28–30). Normally, engagement of these receptors results in prompt proteolytic activation of the downstream caspases including Yama, LAP3, and Mch2. In CrmA-expressing cells, Yama, LAP3, and Mch2 remain as proenzymes, suggesting that CrmA likely inhibits an upstream ICE-like protease such as FLICE (14, 23, 24).

In this study, the apoptotic potential and substrate specificity of recombinant FLICE was determined in a cell-free system. Additionally, FLICE was examined as a potential CrmA target.

**MATERIALS AND METHODS**

**Expression of Recombinant FLICE**—The ICE homology region of FLICE (encoding Ser-217 to Asp-479) was polymerase chain reaction-amplified and subcloned into the bacterial expression vector pET15b (Novagen). Oligonucleotides used for amplification were as follows. Upstream, CAAGAGAACATATGAGTGAATCACAGACTTTGGACAA-AG; downstream, CAGGGATCCTCAATCAGAAGGGAAGACAAGTTT.

The protein was expressed in the BL21 pLy8E Escherichia coli strain and purified using the QiAexpress Kit (Qiagen) following the manufacturer’s instructions.

**25S-Labeled Substrates—**cDNA encoding human ICE, ICH-1L, Tx, ICE-LAP3, ICE-LAP6, FLICE, FLICE2, Mch2, Yama, CrmA, and proIL-1β were subcloned into the mammalian expression vector pcDNA3 (Invitrogen) that is driven in vitro by the T7 promoter. Plasmid templates were used in coupled in vitro transcription/translation reactions to generate [³⁵S]methionine-labeled proteins (Promega).

**Protease Assay—**Cytosolic extracts were prepared from untreated

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The abbreviations used are: caspase, cysteine aspartic acid specific protease; IL-1β, interleukin-1β; ICE, interleukin-1β converting enzyme; TNF, tumor necrosis factor; TNFR-1, tumor necrosis factor receptor 1; CrmA, cytokine response modifier A; LP5, lipopolysaccharide; PARP, poly(ADP-ribose)polymerase; serpin, serine protease inhibitor; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.

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2 C. Vincenz and V. M. Dixit, submitted for publication.
(naive), CD95-stimulated Jurkat cells, or LPS (100 ng/ml)-treated THP-1 cells, as described previously (31). Nuclei were prepared from HeLa cells also as described previously (32). In vitro apoptotic reactions were assayed by the addition of 10^6 HeLa nuclei to 40 μl of extract (6 mg/ml protein concentration), in the presence or absence of recombinant FLICE. In instances where tetrapeptide inhibitors (Bachem) were used, they were added to naive extract 10 min prior to the addition of FLICE. Agarose gel analysis for DNA fragmentation was performed as described previously (31). Immunoblotting of extracts for the large catalytic subunit of LAP3 and Yama and for diagnostic PARP and lamin A cleavage products was carried out as described previously (33). A careful titration was performed to determine the lowest concentration of FLICE sufficient to induce DNA fragmentation and this was then used in all subsequent experiments unless otherwise noted.

In Vitro Binding Assay—50 ng of recombinant FLICE in the presence or absence of 20 μM Ac-DEVD-CHO tetrapeptide inhibitor was incubated with 35S-labeled CrmA in a total volume of 100 μl of buffer (50 mM Hepes, 0.5% CHAPS, 150 mM NaCl, 0.005% bovine serum albumin); an aliquot of each reaction (10 μl) was resolved by SDS-PAGE and subjected to autoradiography to confirm the presence of CrmA. The remaining sample was diluted to 1 ml in binding buffer (0.1% Nonidet P-40, 400 mM NaCl, 0.005% bovine serum albumin, 1 mM EDTA, 50 mM Hepes) and immunoprecipitated with a rabbit polyclonal FLICE (small subunit) antiserum. Precipitates were resolved by SDS-PAGE and analyzed by autoradiography to detect associating CrmA.

Transfection and Immunoprecipitation Analysis—FLICE cDNA epitope-tagged (HA) at the C terminus was co-transfected with a CrmA expression construct into 293 cells. After 48 h, cells (5 x 10^6) were lysed in 1 ml of binding buffer, an aliquot (50 μl) was resolved by SDS-PAGE, and the presence of CrmA was confirmed by immunoblotting. The remaining lysate was immunoprecipitated with a monoclonal HA- epitope tag antibody, and the immune complex was resolved by SDS-PAGE and analyzed by immunoblotting to detect coprecipitating CrmA.

RESULTS AND DISCUSSION

FLICE Induces Apoptosis in a Cell-Free System—A cell free system was utilized to investigate if recombinant FLICE could proteolytically activate caspase zymogens implicated in apoptosis. In most cell types, fragmentation of nuclear DNA into internucleosomal size fragments is the biochemical hallmark of apoptosis. Recombinant FLICE did not induce DNA fragmentation when added directly to indicator HeLa nuclei, suggesting that by itself it was incapable of inducing apoptosis (Fig. 1A). However, in the presence of a cytosolic extract from untreated Jurkat cells (naive extract), it was competent to trigger DNA fragmentation (Fig. 1A). Therefore, essential cytosolic cofactors were required for FLICE to drive the apoptotic reactions to completion. Several independent studies have reported that caspase family members rapidly cleave PARP and lamin A during apoptosis to signature 85- and 40-kDa fragments, respectively (9, 24, 34). Consistent with these studies, FLICE in the presence of naive extract induced cleavage of PARP and lamin A to characteristic apoptotic fragments (data not shown).

Two different tetrapeptide aldehydes based on the cleavage sequence in PARP (Ac-DEVD-CHO) and IL-1β (Ac-YVAD-CHO) irreversibly inhibit select members of the caspase family (8, 35). At nanomolar concentration ranges, Ac-DEVD-CHO attenuates Fas and Yama-induced apoptosis and inhibits Yama-mediated PARP cleavage, whereas Ac-YVAD-CHO inhibits only ICE-mediated IL-1β cleavage (36–38). However, at higher concentrations, Ac-YVAD-CHO will also attenuate ICE- and Fas-induced cell death (28, 38). Both inhibitors were added to naive extracts, and the ability of FLICE to trigger DNA fragmentation was analyzed. As shown in Fig. 1B, Ac-DEVD-CHO potently inhibited FLICE-induced apoptosis, whereas Ac-YVAD-CHO was effective only at higher concentrations, suggesting preference for the DEVD inhibitor. This inhibitor could either be blocking FLICE directly or, alternatively, a downstream caspase family member such as Yama that is susceptible to inhibition by Ac-DEVD-CHO.

FLICE Cleaves Various Members of the Caspase Family of Cysteine Proteases—Activation of caspase family members is tightly regulated and occurs by the proteolytic processing of a single polypeptide-inactive zymogen to an active dimeric species consisting of large and small subunits. All caspases cleave their known substrates following an Asp residue. Indeed, caspase zymogens are themselves activated by cleavage at internal Asp residues that conform to the substrate consensus for the protease family. Not surprisingly, therefore, caspase family members are capable of activating each other, and members have been shown to self-activate when overexpressed in bacteria. It thus appears reasonable to postulate that a cascade of activation occurs in cells upon triggering of the apical death protease. Given this, the activation of nine caspase zymogens was monitored by detecting the emergence of the two-chain active enzyme in cytosolic extracts exposed to recombinant FLICE.

To analyze processing of caspase family members for which antibodies were not available, an assay was developed to monitor processing of radio-labeled zymogen. The validity of such an approach was confirmed by monitoring in parallel the processing of endogenous zymogen and exogenously added radio-zymogen. Tracer amounts of radio-labeled Yama and LAP3 were added to naive extract and processing of endogenous molecules was assessed by immunoblotting and that of exogenous radio-labeled zymogens by autoradiography. As shown in Fig. 2A, identical cleavage products were detected by either method, confirming the validity of using radio-labeled zymogen in instances where antibody reagents are not available. Of note was the exceptional sensitivity of the radiotracer method. Additionally, it was possible to monitor the emergence of both the large and small radiolabeled subunits upon processing. The immunoblotting detection technique was restricted to one or other subunit depending upon the specificity of the antibody reagent.
As shown in Fig. 2, FLICE directly cleaved Yama, LAP3, Tx, LAP6, and FLICE2 in the absence of naive extract; in contrast, Mch2 and ICH1 were efficiently cleaved only in the presence of naive extract. An identical pattern of cleavage products was observed in the presence of apoptotic extracts from CD95 stimulated cells (Ref. 33 and data not shown). Taken together, these results are consistent with the requirement of an intermediary cytosolic component for FLICE-mediated activation of Mch2 and ICH1. The other family members, however, can be directly processed by FLICE. Intriguingly, ICE was not a substrate for FLICE even in the presence of cytosolic extract and was not cleaved by apoptotic extracts from CD95 stimulated cells (data not shown). This was surprising as ICE or a very similar protease has been proposed to be an important component of the CD95 death pathway (39). Prior studies had shown a sequential activation of ICE-like and CPP32-like proteases to occur during this process (38). The ICE zymogen used in the studies was not defective as it was appropriately proteo-
lysically processed by extracts from LPS-stimulated THP-1 cells, a rich source of enzymatically active ICE (data not shown). Taken together, the data suggest that ICE does not participate in FLICE-mediated apoptosis. Consistent with this finding is the recent report that fails to observe processing of pro-ICE during CD95-induced apoptosis (40). A time course study was undertaken to determine the rapidity of processing of the other family members by FLICE in the presence of naive extract. As shown in Fig. 2C, prior to treatment with recombinant FLICE, only the zymogen form of the respective proteases was detectable. However, within 5 min of exposure to FLICE, cleavage products for Yama and Tx were evident. LAP3 and LAP6 processing followed soon thereafter, being visible by 15 min. Only at a relatively late stage (1–2 h) was processing of McI2 and ICH1 observed, supporting the initial conclusion that their activation was not directly mediated by FLICE but rather required an intermediary step (Fig. 2C).

Collectively, these data demonstrate that FLICE is capable of triggering the processing of downstream death proteases, consistent with its being the most apical member of the cascade.

**FLICE, a Potential CrmA Target**—CrmA is a potent inhibitor of serum withdrawal, TNF- and CD95-induced apoptosis (28, 30, 41), and IL-1β release (42). The inhibition of IL-1β release is almost certainly the result of CrmA inhibition of ICE. This abrogates proteolytic maturation of the cytokine, leading to its intracellular retention. The CrmA target responsible for inhibition of apoptosis has been less clearly defined. We previously showed that the CrmA target for apoptosis inhibition was proximal to the downstream effector proteases Yama, LAP3, and McI2 (23, 24), thereby raising the possibility that an upstream protease such as FLICE was the CrmA target. In keeping with this notion, recombinant CrmA blocked FLICE-induced DNA fragmentation of indicator HeLa nuclei in a cell-free system (Fig. 3A). Since it had previously been shown that 15 ng of recombinant CrmA completely blocked the activity of 0.5 ng of recombinant ICE (42), we asked whether a similar ratio of recombinant CrmA to FLICE would be sufficient to block FLICE-induced apoptosis. As shown in Fig. 3B, this ratio completely abrogated the ability of FLICE to cleave caspasezymogens.

To address whether FLICE and CrmA formed a physical complex, FLICE or catalytically inactive FLICE (obtained by prior incubation with Ac-DEVD-CHO) was directly incubated with recombinant CrmA. The putative complex was precipitated using a FLICE antibody, and associating CrmA was detected by autoradiography. As shown in Fig. 3C, only the enzymatically active form of FLICE formed a complex with CrmA. The observed reduction in size of CrmA in the presence of FLICE is explained by the observation that denaturation of serpin-protease complexes results in cleavage of the serpin in the reactive site loop, leading to a corresponding decrease in molecular mass (19, 43). In *vivo* complex formation was demonstrated by transfecting epitope-tagged FLICE and CrmA expression constructs into 293 cells. Total cell lysates were assessed for CrmA expression by immunoblotting prior to immunoprecipitation (*INPUT* in Fig. 3D). CrmA was observed to be present in three forms with the 38-kDa band representing intact CrmA. The 32-kDa form presumably evolved upon cleavage within the reactive site loop on denaturation of the serpin-protease (CrmA-FLICE) complex. Nonspecific proteolysis within the cell lysate was likely responsible for the 26-kDa form. Regardless, upon FLICE immunoprecipitation, the 32-kDa form of CrmA was found to coprecipitate confirming the ability of the two molecules to form a complex in *vivo*. Taken together, the data presented are consistent with FLICE being the most apical member of the protease death cascade and a likely target for the cell death inhibitor CrmA.
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