Molecular Ordering of the Cell Death Pathway

Bcl-2 and Bcl-xL Function Upstream of the CED-3-Like Apoptotic Proteases*

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Genetic analyses of Caenorhabditis elegans has identified three genes that function in the regulation of nematode cell death. Mammalian homologs of two of these genes, ced-9 and ced-3, have been identified and comprise proteins belonging to the Bcl-2 and ICE families, respectively. To date, it is unclear where the negative regulators, ced-9 and bcl-2, function relative to the death effectors, ced-3 and the mammalian ced-3 homologs, respectively. Here, the molecular order of the cell death pathway is defined. Our results establish that Bcl-2 and Bcl-xL function upstream of two members of the ICE/CED-3 family of cysteine proteases, Yama (CPP32/apopain) and ICE-LAP3 (Mch3).

Apoptosis, or programmed cell death, is a physiologic process important in the normal development and homeostasis of multicellular organisms (1, 2). It is encoded by an endogenous program, conserved throughout metazoan evolution, ultimately resulting in cellular suicide. Derangements of apoptosis can have deleterious consequences as exemplified by several human disease states, including acquired immunodeficiency syndrome, neurodegenerative disorders, and cancer (3).

Despite its paramount importance, the molecular mechanism of apoptosis is poorly understood. The nematode Caenorhabditis elegans has been a powerful tool in the identification of critical components of the cell death machinery (4). Systematic genetic analyses have elucidated three genes, ced-3, ced-4, and ced-9, that are important in the regulation of nematode cell death. Mutations of ced-3 and ced-4 abolish all somatic cell deaths that normally occur during the development of C. elegans, suggesting that these genes encode effector components of the pathway (4). By contrast, ced-9 encodes a negative regulator that functions to suppress inappropriate cellular suicide (5). Mammalian homologs of ced-9 and ced-3 have been identified and include proteins belonging to the Bcl-2 and ICE family, respectively (6, 7). However, no homologs of ced-4 have thus far been identified.

Bcl-2 is the prototypic member of a growing family of cell death regulators (8, 9). First identified for its role in B-cell malignancies, expression of Bcl-2 has been shown to inhibit cell death due to a variety of apoptotic stimuli and in numerous cell types (10). Bcl-2 can substitute functionally for ced-9, preventing nematode cell death and further emphasizing the highly conserved nature of the cell death pathway (6).

An important advance came with the discovery that the nematode death effector ced-3 had significant homology with the mammalian protease interleukin-1β-converting enzyme (ICE) (7). ICE is involved in the proteolytic processing of pro-IL-1β to the active cytokine (11, 12). Overexpression of ICE or CED-3 in mammalian cells induces apoptosis, suggesting that ICE, or a related protease, is an essential component of the mammalian cell death pathway (13).

Evidence is growing, however, that ICE itself may not be the mammalian ced-3 equivalent as: 1) a number of cell types stably secrete mature IL-1β without undergoing apoptosis; 2) ICE-deficient mice, although unable to generate active IL-1β, fail to exhibit a prominent cell death defective phenotype (14, 15). Recently, several ICE/CED-3 homologs have been identified which comprise an emerging family of aspartate-specific cysteine proteases that include Nedd-2/CH1 (16, 17), Yama/CPP32/apopain (18–20), TXI/CH2/ICE rel-II (21–23), ICE-rel III (21), Mch2 (24), and ICE-LAP3/Mch3 (25, 26). Distinct from the prototype ICE, Yama and ICE-LAP3 cleave the death substrate poly(ADP-ribose) polymerase (PARP) into signature apoptotic fragments (18, 19, 26). Importantly, CED-3 has a similar substrate specificity as it also cleaves PARP at the same site (27).

Although important regulators of the cell death pathway have been identified, little is known about their molecular order of function. To date, it is unclear whether ced-9 and bcl-2 function upstream or downstream of ced-3 and the mammalian ced-3 equivalent, respectively. Biochemically defining the sequence of the pathway is of utmost importance if one is to gain insight into how the pathway functions and how it is regulated. Here we report that Yama and ICE-LAP3, the two mammalian proteases most related to CED-3, are both activated by apoptotic stimuli. Importantly, we demonstrate order in the pathway by providing biochemical evidence that Bcl-2 and Bcl-xL function upstream of Yama and ICE-LAP3.

MATERIALS AND METHODS

Cell Lines and Culture—Jurkat cells were cultured in complete RPMI 1640 media (10% heat-inactivated fetal bovine serum (HyClone), 1-glutamine, penicillin/streptomycin, and nonessential amino acids). To generate Bcl-2- and Bcl-xL-expressing cell lines, cells were electroporated at 310 V, 960 microfarads in 0.4-cm cuvettes (Bio-Rad) using control vector, pEBS-bcl-2, and pEBS-bcl-xL, and subsequently selected with 0.4 mg/ml hygromycin (Calbiochem). Pooled populations were assessed for expression by immunoblotting using anti-Bcl-xL and anti-Bcl-2 antibodies (Santa Cruz). To generate CrmA expressing) urkat cell lines, cells were similarly transfected using control vector and pZEM-CrmA and selected with 2 mg/ml G418 (LifeTechnologies, Inc.). Clonal lines were derived as described previously (28), and five clones were

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Yama and ICE-LAP3 Are Activated by Various Apoptotic Stimuli—Based on similarities with the structural prototype interleukin-1β converting enzyme, ICE/CED-3 family members are synthesized as proenzymes which are proteolytically processed to form active heterodimeric enzymes (Fig. 1). Since an inducible expression construct (pZEM) was used, vector and CrmA transfectants were incubated with 5 μM CdCl₂ for 4 h before various assays were performed.

Immunoblotting of Yama and ICE-LAP3—Jurkat cells (20 × 10⁶) were left untreated or treated with 2 μM staurosporine (Sigma) or 200 ng/ml anti-APO-1 (IgG3 (29)) for the indicated time periods in a 5-ml volume of serum-free RPMI 1640. Cells were pelleted, sonicated six times for 15 s and then freeze-thawed three times. Samples were centrifuged at 14,000 rpm for 25 min at 4 °C. Cytosolic extracts were removed and added to sample buffer. Samples were analyzed on a 15% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Immunoblotting was done using a 1:1000 dilution of primary antibodies, and a 1:10,000 dilution of secondary anti-rabbit horseradish peroxidase (Amersham), and developed by ECL (Amersham). Rabbit anti-peptide antibodies (Lampire) were raised against the p17 subunit of Yama (NNKFKSTGRMSPTGTD), the p20 subunit of Yama (STAPGGYSWNKDGSG), and the p20 subunit of ICE-LAP3 (PDRSSVFPSLFSKKKN).

DNA Fragmentation Assay and PARP Analysis—PARP analysis was done as described previously (18). Anti-PARP antibody was done C-2-10, which is described previously (30), and recognizes an epitope near the N terminus of PARP located between amino acids 216 and 375. DNA isolation and fragmentation assays were done as described previously (31).

RESULTS AND DISCUSSION

Yama and ICE-LAP3 Are Activated by Various Apoptotic Stimuli—Based on similarities with the structural prototype interleukin-1β converting enzyme, ICE/CED-3 family members are synthesized as proenzymes which are proteolytically processed to form active heterodimeric enzymes (Fig. 1A (11)). Evidence is growing that Yama (CPP32/apopain) may play a more important role in the apoptotic pathway than ICE itself (18, 19). Yama is expressed as a 32-kDa precursor which upon activation is processed into p17 and p12 subunits (19). Therefore, we determined whether Yama is activated as an early event during apoptosis induced by Fas/APO-1 and the protein kinase inhibitor staurosporine (32). Jurkat T-cells expressed the 32-kDa form of Yama, and treatment with staurosporine or anti-APO-1 antibody generated active p17 and p12 subunits (Fig. 1B). Intermediate forms of the larger p17 subunit were also observed and likely contain the small prodomain of Yama (Fig. 1A).

A related member of the ICE/CED-3 family was recently cloned and designated ICE-LAP3/Mch3 (25, 26) Fig. 1A). Of the homologs thus far identified, ICE-LAP3 is the most closely related to Yama, sharing 58% sequence identity and 75% similarity (25). More importantly, endogenous pro-ICE-LAP3 (p35) is also activated by Fas/APO-1 (25) and staurosporine (Fig. 1B), generating p20 and p12 subunits. A 30-kDa form of ICE-LAP3 (p30) was often observed upon activation and likely represents a prodomain-less species.

Taken together, these results show that Yama and ICE-LAP3, the two ICE-like proteases sharing the most sequence homology and substrate specificity with CED-3 (18, 19, 25–27), are activated by an apoptotic signal. In contrast to C. elegans, this suggests that the mammalian cell death pathway may require more than one ICE/CED-3 family member to execute the cell death program.

Bcl-2 and Bcl-xL Function Upstream of Yama and ICE-LAP3—The Bcl-2 family of proteins has been shown to prevent cell death induced by a variety of signals and in numerous cell types (9, 10). However, little is known about where Bcl-2 functions relative to the apoptotic proteases. To address this important question, we generated Jurkat cells overexpressing Bcl-2 or Bcl-xL, using episomal expression constructs. Expression of Bcl-2 and Bcl-xL in the pooled population was confirmed by immunoblotting (Fig. 2A). Stauroporine has been shown to reliably induce Bcl-2-inhibitable cell death in a variety of cell lines (32–34). As expected, Jurkat cells expressing Bcl-2 and Bcl-xL, were resistant to staurosporine-induced apoptosis as assessed by DNA fragmentation (Fig. 2B) and nuclear morphology (data not shown). An apoptotic stimulus that is not consistently blocked by Bcl-2 is activation of Fas/APO-1 (9, 10, 35, 36). In our Jurkat cell system, expression of Bcl-2 or Bcl-xL did not inhibit anti-APO-1-induced cell death or PARP cleavage (Fig. 2, B and C).

To provide biochemical proof of whether Bcl-2 and Bcl-xL function upstream or downstream of the apoptotic ICE/CED-3 cysteine proteases, we directly assessed Yama and ICE-LAP3 activation under various conditions. Overexpression of Bcl-2 or Bcl-xL, abrogated staurosporine-induced generation of active Yama and ICE-LAP3 (Fig. 2D). Similar results were obtained
using another Bd-2/Bd-xL-inhibitable death stimulus, thapsigargin (data not shown), which is a specific inhibitor of the endoplasmic reticulum Ca\(^{2+}\)-ATPase (37). The inhibitory action of Bd-2 and Bd-xL could be "bypassed" by treatment with anti-APO-1 antibody, resulting in activation of the apoptotic proteases (Fig. 2D). Our conclusions are not a peculiarity of Jurkat cells since similar results were obtained using a well characterized Bcl-2-expressing CEM cell line (Ref. 35 and data not shown).

**Evidence for a Proteolytic Cascade in the Fas/APO-1 Cell Death Pathway**—Unlike staurosporine and thapsigargin treatment, Fas/APO-1-induced Yama/ICE-LAP3 activation and resulting apoptosis were not blocked by Bd-2 or Bd-xL in our Jurkat cell system. There could be numerous explanations for this phenomena, including differences between cell types, strength of death stimulus, and expression levels of negative regulators. Alternatively, distinct apoptosis signaling pathways may exist, some of which are more sensitive to Bd-2 and Bd-xL. To address this possibility, we generated Jurkat cells expressing CrmA (Fig. 3A), a poxvirus serpin which a number of groups have shown to abrogate Fas/APO-1-induced apoptosis (28, 38, 39). CrmA is an inhibitor of the ICE family of cysteine proteases and has been shown to inhibit ICE and Yama in vitro (18), CrmA-expressing Jurkat cells were resistant to anti-APO-1-induced cell death, but not staurosporine-induced apoptosis (Fig. 3B). Integrity of PARP was also monitored, and, likewise, CrmA blocked PARP cleavage induced by Fas/APO-1 but not by staurosporine. Similar results were obtained using a previously characterized CrmA-expressing BJ AB cell line (18, 28).

To determine whether CrmA directly inhibited Yama and/or ICE-LAP3 in vivo, activation of these apoptotic proteases was directly assessed. Consistent with DNA fragmentation and PARP analysis (Fig. 3, A and C), staurosporine-induced Yama and ICE-LAP3 activation were not blocked by CrmA (Fig. 3D). Interestingly, Fas/APO-1-induced generation of active Yama protein was also blocked by CrmA (Fig. 3D).
and ICE-LAP3 was potently abrogated by CrmA, suggesting the existence of a CrmA-inhibitable ICE-like protease up-stream of Yama and ICE-LAP3. The fact that staurosporine-induced apoptosis is prevented by Bcl-2/Bcl-xL and not by CrmA, coupled with the observation that Fas/APO-1-induced cell death is inhibited by CrmA but not by Bcl-2/Bcl-xL, argues for the existence of distinct apoptosis signaling pathways.

Conclusions—Prior to this study, little was known about where the negative regulators, ced-9 and ced-2, functioned relative to the death effectors, ced-3 and the mammalian ced-3 homologs (reviewed in Ref. 40). Our results demonstrate that staurosporine-induced cell death, PARP cleavage, and Yama/ICE-LAP3 activation are blocked by Bcl-2 and Bcl-xL. This establishes that Bcl-2 and Bcl-xL function upstream of the two most related mammalian ced-3 homologs, Yama and ICE-LAP3 (Fig. 4). Further, it is reasonable to postulate that a similar relationship exists between ced-9 and ced-3.

In our Jurkat cell system, Fas/APO-1-induced cell death was not blocked by Bcl-2 or Bcl-xL (Fig. 2B). Thus, it was not surprising that Bcl-2 or Bcl-xL failed to inhibit Fas/APO-1-induced PARP cleavage and Yama/ICE-LAP3 activation (Fig. 2, C and D). CrmA, on the other hand, potently abrogated Fas/APO-1-induced cell death, PARP cleavage, and Yama/ICE-LAP3 activation (Fig. 3). Interestingly, CrmA failed to inhibit the death pathway triggered by staurosporine (Fig. 3). Taken together, our data suggest the existence of distinct apoptosis signaling pathways displaying differential sensitivity to Bcl-2/Bcl-xL and CrmA (Fig. 4).

In vitro, CrmA interacts only with the active forms of ICE or Yama (18). However, both Yama and ICE-LAP3 remained as proenzymes in anti-APO-1-treated CrmA expressing cells, suggesting that CrmA inhibits an ICE-like protease upstream of Yama and ICE-LAP3. This hypothesis is especially attractive considering that granzyme B-induced cell death, which is not Bcl-2-inhibitable, likely occurs via direct activation of Yama (and/or Yama-related proteases) (41, 42). Therefore, we postulate that Fas/APO-1 signals the activation of a proteolytic cascade, analogous to the coagulation or complement systems, and in this case, comprised of related ICE/CED-3 family members (Fig. 4).

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