ICE-LAP3, a Novel Mammalian Homologue of the Caenorhabditis elegans Cell Death Protein Ced-3 Is Activated during Fas- and Tumor Necrosis Factor-induced Apoptosis*

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Members of the ICE/ced-3 gene family have been implicated as components of the cell death pathway. Based on similarities with the structural prototype interleukin-1β-converting enzyme (ICE), family members are synthesized as proenzymes that are proteolytically processed to form active heterodimeric enzymes. In this report, we describe a novel member of this growing gene family, ICE-LAP3, which is closely related to the death effector Yama/CPP32/Apopain. Pro-ICE-LAP3 is a 35-kDa protein localized to the cytoplasm and expressed in a variety of tissues and cell lines. Overexpression of a truncated version of ICE-LAP3 (missing the pro-domain) induces apoptosis in MCF7 breast carcinoma cells. Importantly, upon receipt of a death stimulus, endogenous ICE-LAP3 is processed to its subunit forms, suggesting a physiological role in cell death. This is the first report to demonstrate processing of a native ICE/ced-3 family member during execution of the death program and the first description of the subcellular localization of an ICE/ced-3 family member.

Apoptosis, or programmed cell death, is essential for the development and homeostasis of multicellular organisms (1). It is an active form of cellular suicide encoded by an endogenous program that can be triggered by either internal or external cues. The morphological alterations of programmed cell death include cellular shrinkage, membrane blebbing, and chromatin condensation (2). Derangements of apoptosis contribute to the pathogenesis of several human diseases including cancer, acquired immunodeficiency syndrome, and neurodegenerative disorders (3–5).

Despite its biological importance, the molecular mechanism behind apoptosis remains to be defined. Recently, systematic genetic analysis of Caenorhabditis elegans has identified three genes (ced-3, ced-4, and ced-9) that are important in the regulation of nematode cell death. The proteins encoded by ced-3 and ced-4 are required for all somatic cell deaths that occur during the development of C. elegans (6). Mutations of ced-3 and ced-4 abolish the apoptotic capability of cells that normally die during development (7). By contrast, ced-9, which encodes a protein that is homologous to the mammalian gene bcl-2, functions to suppress somatic cell death in the nematode (8). The function of ced-9 can be partially substituted by ectopic expression of bcl-2 (9). These results suggest that components of the apoptotic machinery are conserved throughout evolution and that mammalian counterparts of ced-3 and ced-4 may play an important role in the mammalian cell death pathway. To date, no homologues of ced-4 have been identified. By contrast, numerous relatives of ced-3 have been characterized, comprising a new gene family of cysteine proteases.

The first mammalian homologue of ced-3 identified was interleukin-1β-converting enzyme (ICE) (10), a cysteine protease involved in the processing and activation of pro-interleukin-1β to an active cytokine (11, 12). Overexpression of ICE in Rat-1 cells induced apoptosis, suggesting that ICE may act as the functional mammalian homologue of Ced-3 (13). However, later studies refute this possibility, since ICE-deficient mice develop normally and express no overt defects in apoptosis, except possibly in the Fas pathway (14, 15). Furthermore, apoptotic extracts prepared from chicken DU249 cells failed to cleave the primary substrate of ICE, pro-interleukin-1β (16). Instead, an ICE-like activity in these extracts, termed priICE, cleaved the nuclear enzyme poly(ADP-ribose) polymerase (PARP) into characteristic fragments (16). Purified ICE failed to cleave PARP (16, 17), suggesting that priICE was distinct from ICE. These observations, along with others, suggest that an ICE-like enzyme, rather than ICE itself, plays a role in the mammalian cell death pathway.

Five members of the ICE/Ced-3 family have been recently identified and include Nedd-21/CH1 (18, 19), Yama/CPP32/Apopain (17, 20, 21), TX/ICH2/ICE rel-II (22–24), ICE rel-III (22), and Mch2 (25). All family members share sequence homology with ICE/Ced-3 and contain an active site QACRG pentapeptide in which the cysteine residue is catalytic. Overexpression of these proteins in a variety of cells causes apoptosis.

Among the ICE/Ced-3 family members thus far cloned, evidence is growing that Yama may act as a distal effector of the apoptotic machinery. Yama has been shown to cleave the death substrate PARP, in addition to being inhibitable by the cowpox serpin, CrmA (17). Activated Yama (or Apopain), comprised of p17 and p12 subunits, was purified from cell extracts using a

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U39613.

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The abbreviations used are: ICE, interleukin-1β-converting enzyme; PARP, poly(ADP-ribose) polymerase; PCR, polymerase chain reaction; TNF, tumor necrosis factor; PBS, phosphate-buffered saline.
tetrapeptide aldehyde inhibitor corresponding to amino acids at the PARP cleavage site (21). Depletion of Yama from these extracts abrogated their apoptotic potential in vitro (21). This apoptotic activity could be restored by adding back purified Yama to the depleted extracts (21). Though the evidence is compelling that Yama serves as a functional mammalian homologue of Ced-3, one cannot exclude a role for other ICE/Ced-3 family members in the cell death pathway. For instance, Yama may be the distal effector of a proteolytic cascade that is comprised of (or activated by) other related family members. A precedent for this is the activation of Yama by purified ICE or granzyme B in vitro (17, 26). Alternatively, there may exist a redundant cell death pathway in which individual ICE/Ced-3 family members play a role.

Here we report the cloning and characterization of a novel member of the ICE/Ced-3 family designated ICE-LAP3 (for ICE-Like Apoptotic Protease 3) that is closely related to the death effector Yama. ICE-LAP3 is expressed in a variety of tissues and cell lines. Overexpression of ICE-LAP3 in MCF7 cells induces cell death, and mutation of the putative catalytic cysteine residue abolishes its apoptotic potential. Stimulation of the cytochrome c death receptors, Fas/APO-1 or TNFR-1, triggers processing of pro-ICE-LAP3 to active p20 and p12 subunits. Taken together, our results suggest that ICE-LAP3 is likely a cysteine protease that may have a role in cytokine-mediated cell death.

MATERIALS AND METHODS

Cloning of Human ICE-LAP3—The cDNA corresponding to ICE-LAP3 was identified as a sequence homologous to ICE on searching the Human Genome Sciences private database using established EST methods (27, 28).

DNA Sequencing—The sequence of ICE-LAP3 was confirmed by sequencing plasmid DNA template on both strands by the dideoxy chain termination method using modified T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.). Sequence alignments were performed using DNASTAR Megalign software.

Expression Constructs—The p28 version of ICE-LAP3, which lacks the first 53 N-terminal amino acids that encode the putative prodomain was generated by PCR and subcloned into the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA). The upstream PCR primer (CGGGGTACCGGCCCATGCGGCTGCTACTCATACGTAC) encoded an artificial initiator methionine within Kozak’s consensus (italics) and a custom Kpn restriction site (underlined). The downstream primer (GCTCTAGATGGATGATGCGCTGACGTCG-GAAGATTGTTCC) encoded an AU1 epitope tag (DTYRYI; italics) and an in-frame stop codon (underlined). Alteration of the active site cysteine 184 to alanine was accomplished by site-directed mutagenesis and an in-frame stop codon (underlined). Alteration of the active site cysteine 184 to alanine was accomplished by site-directed mutagenesis and an in-frame stop codon (underlined).

Characterization of ICE-LAP3—The Human Genome Sciences human cDNA data base was searched for genes related to ICE/ced-3. Six cDNAs related to the ICE/ced-3 gene family were isolated, and all except one were previously described (10, 17, 19–25). In this report, we characterize a 2.4-kilobase cDNA that contains 575 bases upstream of the initiation methionine (34) and ending 912 nucleotides later at an Opal codon. Given the presence of an in-frame stop codon 285 base pairs upstream of the initiator methionine and the size of the transcript (2.4 kilobases; see Fig. 2A), it is likely that the full-length coding sequence was identified. This gene encodes a previously undescribed protein of 304 amino acids with a predicted molecular mass of 35 kDa, designated ICE-LAP3 (Figs. 1A, 2).

RESULTS AND DISCUSSION

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ICE-LAP3 Is a Novel Member of the ICE/ced-3 Gene Family—A BLAST search of the GenBank protein data base revealed that the predicted protein sequence of ICE-LAP3 is similar to the C. elegans Ced-3 protein and classifies ICE-LAP3 as a novel member of the ICE/ced-3 gene family. Of the family members thus far cloned, ICE-LAP3 is the most closely related homologue to the mammalian cell death effector, Yama/CPP32/Apoptain (17, 20, 21). ICE-LAP3 has 58% sequence identity (75% similarity) with Yama, 40% identity (57% similarity) with Mch2a, and less than 35% identity (55% similarity) with the other ICE/Ced-3 family members. Like Yama, ICE-LAP3 is highly homologous to Ced-3, sharing 35% sequence identity (54% similarity).

Yama is an Asp-specific cysteine protease responsible for the deavage of the death substrate PARP (17, 21). The full-length
ICE.

...the P1 aspartic acid. These six amino acids are conserved in all peptide (Fig. 1 site defining two subunits that are not separated by a linker...an N-terminal aspartate cleavage site and an internal cleavage...

Comparison with Yama would suggest that ICE-LAP3 contains ICE-LAP3 that corresponds to the active subunits of Yama. The motology between Yama and ICE-LAP3 lies within a region of...

...tive p17 and p12 subunits (21). The majority of sequence homology between Yama and ICE-LAP3 (Fig. 1 C). Yama/Cpp32/Apopain, ICE-LAP3, and Mch2 are closely related to C. elegans Ced-3 and comprise the Yama subfamily. ICE and the ICE-related genes, TX/ICE rel II/CH2 and ICE rel III, form the ICE subfamily, while ICH1 and its mouse homologue, Ned-2, form the Ned-2 subfamily.

Distribution of ICE-LAP3—Northern blot analysis revealed that ICE-LAP3 is constitutively expressed in a variety of fetal and adult human tissues with small amounts in the fetal brain (Fig. 2A). The mRNA transcript is approximately 2.4 kilobases, consistent with the size of the cDNA clones isolated. Using a rabbit anti-peptide antibody directed against ICE-LAP3, a variety of cell lines were examined for expression of endogenous ICE-LAP3 protein, which was detected to a variable extent in all cell lines analyzed (Fig. 2B). Endogenous ICE-LAP3 migrated at approximately 35 kDa, consistent with the predicted molecular mass.

Cellular Localization of ICE-LAP3—Intracellular localization of endogenous ICE-LAP3 was determined using the above described anti-ICE-LAP3 antibody. In Jurkat T cells, ICE-LAP3 localized diffusely to the cytoplasm and juxtamembrane structures (Fig. 2C). Similar results were obtained using 293T cell lines (data not shown). The specificity of this staining was confirmed using preimmune serum and peptide-blocked anti-ICE-LAP3 serum. This is the first reported immunolocalization of an endogenous ICE/Ced-3 family member and is consistent with previous reports suggesting that the death effector machinery resides within the cytoplasm and not in the nucleus (37).

Overexpression of ICE-LAP3 in MCF7 Cells Induces Apoptosis—All ICE/Ced-3 family members have been shown to induce apoptosis when overexpressed in various cell lines. To determine whether ICE-LAP3 may have a role in cell death, an expression construct encoding the full-length protein was transfected into MCF7 breast carcinoma cells and subsequently assessed for apoptotic features. Unlike full-length ICE, expression of full-length ICE-LAP3 failed to induce apoptosis (data not shown). However, expression of a truncated derivative of ICE-LAP3 (pcDNA3 AU1-ICE-LAP3 p28), which lacked 53 N-terminal amino acids corresponding to the putative prodomain, caused cell death (Fig. 3A). The ICE-LAP3 p28-transfected cells displayed morphological alterations typical of adherent cells undergoing apoptosis, becoming rounded, condensed, and detaching from the dish (Fig. 3B). The nuclei of the rounded MCF7 cells exhibited apoptotic morphology as assessed by propidium iodide staining (data not shown). To determine whether amino acid residue Cys385, corresponding to the catalytic Cys385 of ICE, was essential for apoptotic activity, a mutant of ICE-LAP3 was generated in which the cysteine...
residue was altered to an alanine. Overexpression of the mutant form of ICE-LAP3 did not induce apoptotic changes in MCF7 cells (Fig. 3, A and B). Taken together, these results suggest that the activity of ICE-LAP3 is tightly regulated in mammalian cells and that activation of ICE-LAP3 likely requires removal of the pro-domain. This is consistent with the finding that overexpression of the pro-form of Yama (Pro-Yama) in MCF7 cells also failed to induce cell death (data not shown).

ICE-LAP3 Is Activated during Fas- and TNF-induced Apoptosis—Two cell surface cytokine receptors, Fas/APO-1 and the p55 receptor for tumor necrosis factor (TNFR-1), have been shown to trigger apoptosis by their respective natural ligands or specific agonist antibodies (30). To determine whether ICE-LAP3 may be a component of the Fas- and TNF-induced cell death pathways, concomitant processing of endogenous pro-ICE-LAP3 was assessed. Over a time course of incubation with anti-Fas antibody, pro-ICE-LAP3 was processed to form p20 and p12 subunits in both Jurkat T cells and BJAB B cells (the anti-ICE-LAP3 antibody generated was directed against the p20 subunit, which could therefore be detected) (Fig. 3C). Similar results were observed during TNF-induced cell death of MCF7 cells (Fig. 3D). CrmA, a poxvirus gene product, has been shown to potently block both Fas- and TNF-induced cell death (30). Engagement of Fas and TNFFR in CrmA-expressing cells abrogated ICE-LAP3 activation as well as cell death (Fig. 3D) (30). As expected, ICE-LAP3 was processed in inactive mutant CrmA (17) expressing cell lines (Fig. 3D).

In conclusion, we have identified a novel death-inducing protein of the ICE/Ced-3 family. ICE-LAP3 was immunolocalized to the cytoplasm, where the death effector machinery is thought to reside (37). This is the first report describing the activation of an endogenous ICE/Ced-3 cysteine protease during Fas- and TNF-induced cell death, suggesting that it is a mediator of the cytokine-mediated cell death pathway. Further, there is compelling evidence that a family member, Yama, acts as a distal effector of the cell death pathway (17, 21). The high homology between Yama and ICE-LAP3 is intriguing and raises many important questions about a potential role of ICE-LAP3 in a general cell death program. One possibility is that ICE-LAP3 also acts as an effector in a redundant cell death pathway distinct from the one utilizing Yama. Alternatively, ICE-LAP3 could act as an upstream enzyme, or “Yama converter,” responsible for the processing of Pro-Yama to an active death protease. The cloning and characterization of the Yama-related protein ICE-LAP3 will help address these specific questions and, in general, enhance our understanding of the cell death machinery and the proteases that compose it.

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