

ICE-LAP6, a Novel Member of the ICE/Ced-3 Gene Family, Is Activated by the Cytotoxic T Cell Protease Granzyme B*

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Members of the ICE/Ced-3 gene family are likely effector components of the cell death machinery. Here, we characterize a novel member of this family designated ICE-LAP6. By phylogenetic analysis, ICE-LAP6 is classified into the Ced-3 subfamily which includes Ced-3, Yama/CPP32/apopain, Mch2, and ICE-LAP3/Mch3/CMH-1. Interestingly, ICE-LAP6 contains an active site QACGG pentapeptide, rather than the QACRG pentapeptide shared by other family members. Overexpression of ICE-LAP6 induces apoptosis in MCF7 breast carcinoma cells. More importantly, ICE-LAP6 is proteolytically processed into an active cysteine protease by granzyme B, an important component of cytotoxic T cell-mediated apoptosis. Once activated, ICE-LAP6 is able to cleave the death substrate poly(ADP-ribose) polymerase into signature apoptotic fragments.

Apoptosis, or programmed cell death, is a physiologic process important in the normal development and homeostasis of metazoans (1). It is becoming apparent that a class of cysteine proteases homologous to *Caenorhabditis elegans* Ced-3 play the role of "executioner" in the apoptotic mechanism (2–4). In the nematode, two proteins, encoded by *ced-3* and *ced-4*, are required for all somatic cell deaths that occur during development (5). Mutations of *ced-3* and *ced-4* abolish the apoptotic capability of cells that normally die during *C. elegans* embryogenesis (6). While no mammalian homologs of *ced-4* have been identified, *ced-3* shares sequence similarity with interleukin-1 β converting enzyme (ICE)¹ (7), a cysteine protease involved in the processing and activation of pro-interleukin-1 β to an active cytokine (8, 9). Recently, numerous homologs of ICE/Ced-3 have been characterized, comprising a new gene family of cysteine proteases.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U567390.

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¹ The abbreviations used are: ICE, interleukin-1 β converting enzyme; PARP, poly(ADP-ribose) polymerase; PCR, polymerase chain reaction; ICE-LAP6, ICE-like apoptotic protease 6; PAGE, polyacrylamide gel electrophoresis; X-gal, 5-bromo-4-chloro-3-indolyl β -[d-galactoside]; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

To date, seven members of the ICE/Ced-3 family have been identified and include ICE (8), TX/ICH2/ICE rel-II (10–12), ICE rel-III (10), ICH1/Nedd-2 (13, 14), Yama/CPP32/Apopain (15–17), Mch2 (16), and ICE-LAP3/Mch3/CMH-1 (18–20). All family members share sequence homology with ICE/Ced-3 and contain an active site QACRG pentapeptide in which the cysteine residue is catalytic. Ectopic expression of these proteases in a variety of cells causes apoptosis. Phylogenetic analysis of the ICE/Ced-3 3gene family revealed three subfamilies (3, 18). Yama, ICE-LAP3, and Mch2 are closely related to *C. elegans* Ced-3 and comprise the Ced-3 subfamily. ICE and the ICE-related genes, ICE rel-II, and ICE rel-III form the ICE subfamily, while ICH1 and its mouse homologue, NEDD-2 form the NEDD-2 subfamily.

Based on similarities with the structural prototype interleukin-1 β converting enzyme, ICE/Ced-3 family members are synthesized as zymogens that are capable of being processed to form active heterodimeric enzymes (9). It will be important to determine which family members are in fact activated in response to apoptotic stimuli. Previous studies have demonstrated that pro-Yama and pro-ICE-LAP3 are processed into active subunits in response to various death stimuli including engagement of Fas/APO-1 or treatment with staurosporine (18, 21). Further, the serine protease granzyme B, one of the major effectors of cytotoxic T cell-mediated apoptosis, was shown to directly activate Yama (but not ICE), *in vitro* (22, 23).

Here we report the cloning and characterization of a novel member of the ICE/Ced-3 gene family designated ICE-LAP6 (for ICE-Like Apoptotic Protease 6). Based on sequence homology, ICE-LAP6 is classified in the subset of family members most related to *C. elegans* Ced-3 including Yama, ICE-LAP3, and Mch2. Interestingly, ICE-LAP6 contains a unique active site pentapeptide (QACGG rather than QACRG), which distinguishes it from other family members. Overexpression of ICE-LAP6 in MCF7 breast carcinoma cells induces cell death and mutation of the putative catalytic cysteine residue abolishes its apoptotic potential. Furthermore, granzyme B directly activates ICE-LAP6 and Yama *in vitro*, suggesting that granzyme B may mediate its cytotoxic effect via activation of several ICE/Ced-3 family members. Once activated, Yama and ICE-LAP6 are both able to cleave the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) into signature apoptotic fragments. Taken together, our results suggest that ICE-LAP6, like other members of the Ced-3 subfamily, may have an important role in the apoptotic mechanism.

MATERIALS AND METHODS

Cloning of Human ICE-LAP6—The cDNA corresponding to the partial open reading frame of ICE-LAP6 was identified as a sequence homologous to ICE-LAP3 (18) on searching the Human Genome Sci-

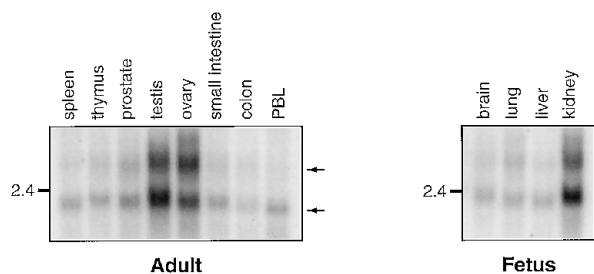


FIG. 2. **Tissue distribution of ICE-LAP6.** A, a human adult and fetal tissue poly(A)⁺ Northern blot (Clontech) was probed with ³²P-labeled ICE-LAP6 cDNA. PBL, peripheral blood leukocyte; arrows indicate the two ICE-LAP6 mRNA transcripts (2.3 and 3.0 kilobases).

out at 37 °C in 10 mM dithiothreitol. Samples were analyzed by immunoblotting with anti-PARP monoclonal antibody C-2-10 as described previously (15).

RESULTS AND DISCUSSION

Cloning of ICE-LAP6—The Human Genome Sciences human cDNA data base was searched for genes related to the ICE-LAP3 peptide sequence (18). A novel cDNA clone, encoding a partial open reading frame, was identified and showed sequence homology with members of the ICE/Ced-3 gene family. To obtain a full-length cDNA, a human chronic myelogenous leukemia cell (K562) cDNA library was screened. Of 22 positive clones, 6 clones yielded a 2.3-kilobase cDNA containing an 1252-base pair open reading frame that encoded a novel protein with a predicted molecular mass of 45.8 kDa, designated ICE-LAP6 (Fig. 1A). The putative initiator methionine (GCCATGG) was in agreement with the consensus Kozak's sequence for translation initiation (30).

ICE-LAP6 Is a Novel Member of the ICE/Ced-3 Gene Family—A BLAST search of GenBank protein data base revealed that the predicted protein sequence of ICE-LAP6 has significant similarity to the members of the ICE/Ced-3 family, particularly in the regions corresponding to the active subunits of ICE (9). In this region, ICE-LAP6 shares 31% sequence identity (55% sequence similarity) with the *C. elegans* Ced-3 protein, 33% identity (52% sequence similarity) with ICE-LAP3, 30% identity (56% similarity) with Mch2 α , and 29% sequence identity (52% similarity) with Yama. ICE-LAP6 also has 25–28% sequence identity with ICE and the ICE-related genes, ICE rel II and ICE rel III. Phylogenetic analysis of the ICE/Ced-3 gene family showed that ICE-LAP6 is a member of the Ced-3 subfamily which includes Yama, ICE-LAP3, and Mch2 (Fig. 1B). Like Ced-3, ICE-LAP6 contains a long N-terminal putative prodomain.

Based on the x-ray crystal structure of ICE (31, 32), the amino acid residues His²³⁷, Gly²³⁸, and Cys²⁸⁵ of ICE are involved in catalysis, while the residues Arg¹⁷⁹, Gln²⁸³, and Arg³⁴¹ form a binding pocket for the carboxylate side chain of the P₁ aspartic acid. These six residues are conserved in all ICE/Ced-3 family members thus far cloned as well as in ICE-LAP6. However, residues that form the P₂–P₄ binding pockets are not widely conserved among family members, suggesting that they may determine substrate specificity. Interestingly, ICE-LAP6 contains a unique active site pentapeptide QACGG, instead of the QACRG shared by other family members (Fig. 1C).

Distribution of ICE-LAP6—Northern blot analysis revealed that ICE-LAP6 is constitutively expressed in a variety of fetal and adult human tissues (Fig. 2). Two ICE-LAP6 mRNA transcripts were detected (Fig. 2). The 2.3-kilobase transcript corresponds to the size of the cDNA clones isolated from the K562 library. The other transcript, which is approximately 3 kilo-

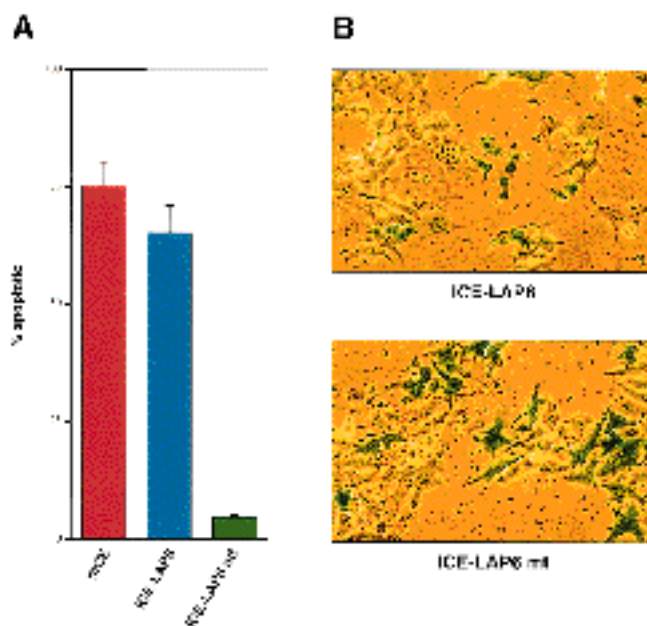


FIG. 3. **Overexpression of ICE-LAP6 induces cell death in mammalian cells.** A, MCF7 breast carcinoma cells were transiently transfected with the reporter gene β -galactosidase and either C-terminal flag-tagged ICE-LAP6, the mutant version with the catalytic cysteine residue inactivated (ICE-LAP6 mt), or ICE as described under "Materials and Methods." Percent apoptotic cells represents the mean value from three independent experiments (mean \pm S.D.). B, Transfected cells were stained with X-gal and examined by phase contrast microscopy.

bases, may represent an alternatively spliced ICE-LAP6 isoform.

Overexpression of ICE-LAP6 in MCF7 Cells Induces Apoptosis—To study the functional role of ICE-LAP6, we transiently transfected MCF7 breast carcinoma cells with an expression vector encoding the full-length ICE-LAP6 protein (ICE-LAP6-flag) and subsequently assessed for apoptotic features. Like the other ICE/Ced-3 family members, expression of ICE-LAP6 caused cell death (Fig. 3A). The ICE-LAP6-transfected MCF7 cells displayed morphological alterations typical of adherent cells undergoing apoptosis, becoming rounded, condensed, and detaching from the dish (Fig. 3B). ICE-LAP6 induced apoptosis was inhibited by the broad spectrum ICE inhibitor z-VAD fmk (33) (data not shown). To determine whether the amino acid residue Cys²⁸⁶, corresponding to the catalytic Cys²⁸⁵ of ICE, was essential for apoptotic activity, a mutant form of ICE-LAP6 was generated in which the cysteine residue was altered to an alanine. As predicted, overexpression of the mutant form of ICE-LAP6 did not induce apoptotic changes in MCF7 cells (Fig. 3, A and B). Furthermore, these results demonstrate that an ICE/Ced-3 family member containing an active site QACGG pentapeptide (rather than QACRG) may still possess apoptosis-inducing potential and presumably enzymatic activity.

Proteolytic Activation of ICE-LAP6 by Granzyme B—Members of the ICE/Ced-3 gene family are synthesized as proenzymes and activated by proteolytic cleavage at specific aspartate residues to form heterodimeric enzymes. In ICE, this cleavage removes the prodomain and produces a heterodimeric complex consisting of p20 and p10 subunits (9). Similarly, activated Yama is comprised of two subunits, p17 and p12, which are derived from a 32-kDa proenzyme (17). The mechanism by which death signals activate ICE/Ced-3 family members is poorly understood. Recent studies on granzyme B, however, suggest that cytotoxic T cells may utilize this secreted serine protease to directly activate members of the ICE/Ced-3

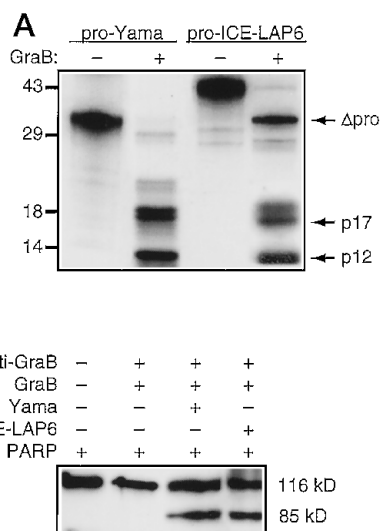


FIG. 4. ICE-LAP6 is activated by the cytotoxic T cell protease granzyme B. *A*, Yama and ICE-LAP6 are proteolyzed by granzyme B *in vitro*. Purified *in vitro*-translated pro-Yama or pro-ICE-LAP6 was incubated with buffer or granzyme B as described under "Materials and Methods." After incubation, 20 μ l of the reactions were analyzed by SDS-PAGE and autoradiography. Δ pro indicates an intermediate of ICE-LAP6 and is presumed to correspond to a form of ICE-LAP6 in which the prodomain is removed. *p17* and *p12* indicate the active subunits of Yama and ICE-LAP6. *B*, granzyme B-processed ICE-LAP6 cleaves PARP to an 85-kDa apoptotic fragment. After incubation with granzyme B (*GraB*) as described above, anti-*GraB* was added to the rest of the reaction mix to neutralize granzyme B activity. Following a 15-min incubation, purified PARP was added, and the reaction was allowed to proceed for 2 h. Samples were analyzed by SDS-PAGE and subsequent immunoblotting with anti-PARP monoclonal antibody C-2-10.

family. It has been demonstrated that granzyme B can proteolytically activate pro-Yama, generating an active enzyme capable of cleaving the death substrate PARP into characteristic fragments (22, 23). By contrast, ICE, although cleaved by granzyme B, fails to be activated (22).

Thus, we determined whether ICE-LAP6 can serve as a substrate for granzyme B. His₆ tagged ICE-LAP6 and Yama were generated by *in vitro* transcription/translation, and subsequently purified by Ni-affinity chromatography as described under "Materials and Methods." The purified *in vitro* translated pro-ICE-LAP6 or pro-Yama was incubated with purified granzyme B (34, 35). After 4 h at 37 °C, ICE-LAP6 was proteolytically processed into three fragments. The two low molecular weight bands represent the active subunits of ICE-LAP6 and correspond to the p17 and p12 subunits of active Yama (Fig. 4A). The doublet at 17 kDa is likely generated by differential cleavage following either of the two aspartic acid residues that are distal to the QACGG motif and are circled in Fig. 1A. The 32-kDa band is an likely intermediate, in which only the prodomain is removed (a similar intermediate is generated in the activation of ICE-LAP3) (18, 21). Next, we assessed whether granzyme B-mediated cleavage of ICE-LAP6 generates an active enzyme by assaying for PARP cleavage. PARP is proteolyzed during many forms of apoptosis, and the enzyme(s) responsible is likely of the ICE/Ced-3 family. To exclude the possibility of direct cleavage of PARP by granzyme B, granzyme B-processed ICE-LAP6 and Yama were incubated with a selective inhibitor of granzyme B (anti-*GraB*) as described previously (22). Interestingly, both granzyme B-processed Yama and ICE-LAP6 were active as determined by their ability to cleave PARP (Fig. 4B). Unlike ICE, ICE-LAP6 and other members of the Ced-3 subfamily are able to cleave the PARP into signature apoptotic fragments (15–17, 19, 20, 36).

In conclusion, we have identified a novel member of the ICE/Ced-3 family of cysteine proteases. ICE-LAP6 has a unique active site QACGG pentapeptide and is classified in the subfamily most related to Ced-3 and Yama. Ectopic expression of ICE-LAP6 in mammalian cells causes apoptosis. Importantly, ICE-LAP6, like Yama, was directly activated by granzyme B *in vitro*, suggesting that cytotoxic T cells may mediate apoptosis by activating more than one ICE/Ced-3 family member in susceptible target cells. Yama, ICE-LAP3, and now ICE-LAP6, have been shown to be proteolytically activated by apoptotic stimuli. The cloning and characterization of ICE-LAP6 will enhance our understanding of the cell death machinery and the proteases that compose it. Additionally, it will be necessary to develop specific inhibitors for each of the three functionally indistinguishable ICEs (ICE-LAP6, ICE-LAP3 and Yama) or inactivation of their genes by homologous recombination before one can discern the contribution of each to granzyme B mediated apoptosis.

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