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-1β converting enzyme (ICE) (7), a cysteine protease involved in the processing and activation of pro-interleukin-1β to an active cytokine (8, 9). Recently, numerous homologs of ICE/Ced-3 have been characterized, comprising a new gene family of cysteine proteases.

To date, seven members of the ICE/Ced-3 family have been identified and include ICE (8), TX/CH2/ICE rel-III (10–12), ICE rel-I (10), ICH1/Nedd-2 (13, 14), Yama/CPP32/apopain (15–17), Mch2 (16), and ICE-LAP3/Mch3/CMH-1 (18–20). All ICE/Ced-3 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 gene 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-1β converting enzyme, ICE/Ced-3 family members are synthesized aszymogens 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 Sc-
Characterization of ICE-LAP6

Fig. 1. Sequence analysis of ICE-LAP6. A, predicted amino acid sequence of ICE-LAP6. The active site pentapeptide QACGG is underlined. Putative Asp cleavage sites are indicated with an asterisk. B, phylogenetic analysis of the ICE/Ced-3 gene family. C, sequence alignment of known members of the mammalian ICE/Ced-3 family and the C. elegans gene Ced-3. The pentapeptide QACRG is boxed. Based on the x-ray crystal structure of ICE, the conserved residues involved in catalysis are marked with filled circles; filled triangles represent the binding pocket for the carboxylate of the P$_i$ Asp; filled squares indicate the residues adjacent to the P$_i$-P$_j$ amino acids.

Apoptosis Assay—MCF7 breast carcinoma cells were transiently transfected as described previously (28). Briefly, 2.5 × 10$^5$ MCF7 cells were transfected with 0.25 μg of the reporter plasmid pCMV β-galactosidase plus 1 μg of test plasmid in 6-well tissue culture dishes using Lipofectamine (Life Technologies, Inc.) and after 5 h, 1 ml of serum-containing growth media was added. Two days later, the cells were fixed with 0.5% glutaraldehyde and stained with X-gal for 4 h. Cells were visualized by phase-contrast microscopy. At least 300 β-galactosidase-positive cells were counted for each transfection (n = 3) and identified as apoptotic or nonapoptotic based on morphological alterations typical of adherent cells undergoing apoptosis including becoming rounded, condensed, and detaching from the dish (29).

Expression and Purification of His$_6$-tagged Yama and His$_6$-tagged ICE-LAP6—35S-Labeled Yama and ICE-LAP6 proteins were generated by in vitro transcription/translation using the TNT kit (Promega) according to the instructions of the manufacturer; the template plasmids were ICE-LAP6 His and Yama His (15). The translated proteins were purified by chromatography as described previously (15).

Activation of ICE-LAP6 and Yama by Granzyme B—In vitro translated pro-ICE-LAP6 or pro-Yama was activated by incubation with granzyme B as described previously (22). Briefly, 48 μl of 35S-labeled protein were incubated with 20 pmol of purified granzyme B (22) in a total volume of 50 μl. After 4 h, 20 μl of reaction was removed for SDS-PAGE analysis. 520 pmol of anti-GraB (22) was added to the rest of the reaction mix to neutralize granzyme B activity. Following a 15-min incubation, 1 μl (150 ng) of purified PARP (15) was added, and the reaction was allowed to proceed for 2 h. The control reaction containing PARP alone or PARP plus granzyme B and anti-GraB was carried out under identical conditions, except that Yama or ICE-LAP6 was not added. The reaction buffer contained 50 mM Hapes (pH 7.4), 0.1 mM NaCl, 0.1% CHAPS, and 10% sucrose. All incubations were carried...
the P1 aspartic acid. These six residues are conserved in all
library. The other transcript, which is approximately 3 kilo-
residues, may represent an alternatively spliced ICE-LAP6
bases, may represent an alternatively spliced ICE-LAP6

RESULTS AND DISCUSSION

Cloning of ICE-LAP6—The Human Genome Sciences hu-
man 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 se-
quence 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
dones, 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 Fami-
ly—A BLAST search of GenBank protein data base revealed
that the predicted protein sequence of ICE-LAP6 has signifi-
cant similarity to the members of the ICE/Ced-3 family, par-
ticularly 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 Mch2α, and 29% sequence ident-
ty (52% similarity) with Yama. ICE-LAP6 also has 25–31% se-
quence 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 sub-
family 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 P4 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 P1–P3 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 QAC-
GG, 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 tran-
scripts were detected (Fig. 2). The 2.3-kilobase transcript cor-
responds to the size of the cDNA clones isolated from the K562
library. The other transcript, which is approximately 3 kilo-

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).

FIG. 3. Overexpression of ICE-LAP6 in MCF7 Cells Induces Apopto-
sis—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 Cys285, 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. 3A, 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 apop-
Tisso-inducing potential and presumably enzymatic activity.

Proteolytic Activation of ICE-LAP6 by Granzyme B—Mem-
ers of the ICE/Ced-3 gene family are synthesized as proen-
zymes and activated by proteolytic cleavage at specific aspar-
tate 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 mecha-
nism by which death signals activate ICE/Ced-3 family mem-
bers is poorly understood. Recent studies on granzyme B, how-
ever, suggest that cytotoxic T cells may utilize this secreted
serine protease to directly activate members of the ICE/Ced-3

out at 37 °C in 10 mM dithiothreitol. Samples were analyzed by immu-
noblotting with anti-PARP monoclonal antibody C-2–10 as described
previously (15).
Characterization of ICE-LAP6

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 apoptosis 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 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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