ERICE, a Novel FLICE-activatable Caspase*

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Programmed cell death, or apoptosis, is a process of fundamental importance to cellular homeostasis in metazoan organisms (Ellis, R. E., Yuan, J., and Horvitz, H. R. (1991) Annu. Rev. Cell Biol. 7, 663–698). The caspase family of mammalian proteases, related to the nematode death protein CED-3, plays a crucial role in apoptosis and inflammation. We report here the isolation and characterization of a new caspase, tentatively termed ERICE (Evolutionarily Related Interleukin-1β Converting Enzyme). Based on phylogenetic analysis, ERICE (caspase-13) is a member of the ICE subfamily of caspasases which includes caspase-1 (ICE), caspase-4 (ICEap-II, TX, ICH-2), and caspase-5 (ICErel-III, TY). Overexpression of ERICE induces apoptosis of 293 human embryonic kidney cells and MCF7 breast carcinoma cells. Like other members of the subfamily, ERICE is not activated by the serine protease granzyme B, a caspase-activating component of cytotoxic T cell granules. Therefore, ERICE most likely does not play a role in granzyme B-induced cell death. ERICE, however, was activated by caspase-8 (FLICE, MACH, Mch-5), the apical caspase activated upon engagement of death receptors belonging to the tumor necrosis factor family. This is consistent with a potential role for ERICE in this receptor-initiated death pathway.

Apoptosis, or programmed cell death, is an evolutionarily conserved process central to the normal development and homeostasis of multicellular organisms (1). Genetic analysis of the nematode Caenorhabditis elegans has revealed three core components of the death pathway, ced-3, ced-4, and ced-9 (2). ced-3 and ced-4 are death genes, and mutations in either attenuate the elimination of cells that normally die during worm development. These central players of cell death in the nematode are conserved in vertebrates. The CED-3 molecule was found to be homologous to the mammalian cysteine protease (ced-3), and these central players of cell death in the nematode have a proven role in inflammation or apoptosis. Members include ICE (caspase-1) (3, 4), ICH-1 (caspase-2) (8), CPP32, apopain, Yama (caspase-3) (9–11), ICEap-II, TX, ICH-2 (caspase-4) (12–14), ICErelIII, TY (caspase-5) (12), Mch2 (caspase-6) (15), Mch3, ICE-LAP3, CMH-1 (caspase-7) (16–18), FLICE, MACH, Mch5 (caspase-8) (19–21), ICE-LAP6, Mch6 (caspase-9) (22–24), and Mch4 (caspase-10) (25, 26). Two more murine caspasases with no known human counterparts have been described mICH-3, mCASP-11 (caspase-11) (26, 27) and mICH-4, mCASP-12 (caspase-12) (27, 28). All members of the caspase family share a number of amino acid residues crucial for substrate binding and catalysis (29, 30). For caspase-1, these residues include Cys285 and His237, which hydrogen bond with the thiohemiacetal of the enzyme-substrate complex, and Gly238, which forms the oxyanion hole that stabilizes the oxy-anion of the reaction intermediates. Arg179, Gln283, Arg241, and Ser247 form the binding pocket for the carboxylate side chain of the P1-aspartic acid.

Although the mechanism of their protease action is conserved, differences in primary sequences account for differential substrate specificity. The caspase family has been divided into three groups based upon substrate specificity using a positional scanning substrate combinatorial library (31). The most divergent amino acid substitution occurs at substrate position P4. Caspases-2, -3, and -7, and CED-3 have a preference for the motif DExD, and caspases-6, -8, and -9 display a specificity for (I/V/L)ExD. Members of these two subfamilies play a prominent role in cell death. In contrast, caspases 1, 4, and 5, which belong to the ICE subfamily and possess a WEHD \ substrate specificity, appear to play a primary role in cytokine maturation and inflammation. Hydrophobic amino acids are not found in the P4 position of substrates known to be cleaved during cell death (31).

Caspase inhibitors have been constructed based upon the presumed substrate specificity of the different caspases. The first caspase-1 subfamily specific inhibitor was based upon the tetrapeptide recognition sequence YVAD present in prointerleukin-1β, a natural substrate for caspase-1. Similarly, a tetrapeptide inhibitor was designed around the putative PARP cleavage site, DEVD, to be selective for the proapoptotic caspases (32). Aldehyde derivatives of these inhibitors are reversible and form a thiohemiacetal with the active site cysteine. Chloromethyl, fluoromethyl, acyloxymethyl, diazoethyl, α-pyrazoloxymethyl, and phosphonyloxymethyl ketones function as irreversible inhibitors by covalently forming a thiomethyl ketone to the sulfur atom of the active site cysteine in the large subunit (30). In vitro data show Ac-YVAD-aldehyde to be a potent inhibitor of caspase-4 and caspase-1 but ineffective in inhibiting caspase-3 and caspase-7. In contrast, caspase-3

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15702
and caspase-7 are potently inhibited by Ac-DEVD-CHO (33). Not surprisingly, a number of pathogenic viruses also encode caspase inhibitors as a means of attenuating immune surveillance mechanisms that work by inducing apoptosis of infected cells. For example, the pox virus cytokine response modifier protein CrmA inhibits caspase-1 and -8 most effectively (11, 34), whereas the baculovirus protein p35 inhibits numerous activated caspases (6, 35).

Caspases exist as single polypeptidezymogens composed of a prodomain of variable length plus a large subunit (which contains the catalytic cysteine) and a small subunit (for reviews, see Refs. 36–40). These subunits are flanked by Asp-X sites, suggesting that activation is mediated by an aspartate-specific protease. All caspases require accurate processing at the internal aspartate residues to generate an active dimeric enzyme. Activated members of the caspase family function as the effector arm of the cell death pathway by cleaving a growing number of cellular substrates including laminas (41–44), kinases (45, 46), DNA fragmentation factor (47), keratin-18 (48), FAK (49), as well as other caspases.

Granzyme B, an aspartate-directed serine protease employed by cytotoxic T cells to kill target cells (50), is the only other enzyme known to cleave and activate multiple caspase members involved in apoptosis including caspase-2 (51), -3 (52, 53), -6 (42), -7 (54, 55), -8 (19), -9 (22), and -10 (21). Analysis of the sites of cleavage of caspase-3, -7, and -10 indicates that granzyme B preferentially cleaves at the LXXD sequence found at the COOH-terminal end of the large subunit. Initial cleavage at this site has been shown to be essential for productive processing of caspase zymogens (21, 55, 56). However, granzyme B will not productively process members of the ICE subfamily in keeping with the less prominent role for this subfamily in apoptosis (57).

Caspase-8 is the initiating caspase in the apoptotic cascade that is activated by engagement of death receptors belonging to the tumor necrosis factor receptor family. Caspase-8 contains two NH2-terminal tandem repeats within the prodomain which are homologous to the death effector domain in the adaptor molecule FADD and allow for its recruitment to the receptor signaling complex. The remainder of the molecule is highly similar to the CED-3 subfamily of caspases (19, 20). In a cell-free system, caspase-8 has been shown to process a variety of proapoptotic caspases; but like granzyme B, it did not activate members of the ICE subfamily (34).

We report here the cloning and characterization of a novel member of the ICE subfamily termed ERICE for Evolutionarily Related ICE (caspase-13). Based upon sequence identity, it is most related to caspase-4 and caspase-5. Overexpression of ERICE in MCF7 and 293 cells induces apoptosis that can be blocked by p35 or CrmA. As expected, mutation of the catalytic cysteine abolishes its proapoptotic activity. In keeping with it being a member of the ICE subfamily, ERICE is not activated by granzyme B. However, unlike other characterized members of the ICE subfamily, ERICE is activated by caspase-8, consistent with a potential role in apoptosis.

MATERIALS AND METHODS

Cloning of ERICE—A database containing more than 1 million ESTs (expression sequence tags) was generated through the combined efforts of Human Genome Sciences and the Institute for Genomic Research using high throughput automated DNA sequence analysis of randomly selected human cDNA clones (50, 55). Sequence homology comparisons of each EST were performed against the GenBank data base using the BLAST and BLASTN algorithms (60). ESTs having homology to previously identified sequences (probability equal to or less than 0.01) were given a tentative designation based on the identity of the sequence. A specific homology and motif search using the known amino acid and DNA sequence of caspase-4 (12) revealed an EST possessing >50% homology to caspase-4. A full-length clone (HFJAB36) representative of the original EST was obtained by screening a human skin fibroblast cDNA library.

Expression Vectors—Expression constructs encoding full-length ERICE were made with a COOH-terminal hemagglutinin tag. Alteration of the active site cysteine to alanine was accomplished by site-directed mutagenesis employing a four-primer two-step polymerase chain reaction protocol as described previously (61). Alteration of potential aspartate cleavage sites was performed following the QuikChange site-directed mutagenesis kit (Strategene). The fidelity of polymerase chain reaction for full-length and mutant expression constructs was confirmed by sequence analysis.

Apoptosis Assays—Cell death assays were performed as described (62).

Northern Blot Analysis—Adult and fetal human multiple tissue Northern blots (CLONTECH) containing 2 μg/lane poly(A)+ RNA were hybridized according to the manufacturer’s instructions, using a 52P-labeled BamHI/PovII 400-base pair restriction fragment containing most of the ERICE homology. Cleavage Assays and Activation—In vitro translated 35S-labeled ERICE and its mutants were treated using either granzyme B or recombinant caspase-8 as described previously (22, 34, 53). To determine whether ERICE was productively processed on exposure to caspase-8 or granzyme B, radiolabeled ERICE was incubated with biotinylated YVAD chloromethyl ketone as described previously (34, 63) and active site-labeled protease precipitated with streptavidin-agarose. Streptavidin beads were washed three times with biotinylation buffer (20 mM Tris, pH 8.0, 0.1% CHAPS, 2 mM dithiothreitol), and bound protease was eluted by boiling in sample buffer, resolved on a 10–20% acrylamide gel, and visualized by autoradiography. The cleavage boundaries were mapped using ERICE mutants in which candidate aspartate residues were changed to alanine. For in vivo cleavage studies, mutant ERICE was cotransfected with FLICE or Fas with or without CrmA. Lysates were made with 500 μl of lysis buffer as described previously (19). Immunoblotting was performed with anti-hemagglutinin or anti-FLAG antibodies.

RESULTS AND DISCUSSION

ERICE Is a Novel Member of the Caspase Gene Family—The full-length 2.2-kilobase ERICE cDNA contained an open reading frame of 377 amino acids encoding a protein of predicted molecular mass 43.0 kDa (Fig. 1A). A BLAST search of the GenBank protein sequence data base revealed ERICE to possess significant homology to other members of the caspase family, particularly the ICE subfamily, which includes caspase-1, caspase-4, caspase-5, and murine caspases-11 and -12 (Fig. 1B). ERICE has 75% sequence identity to caspase-4, 61% sequence identity to caspase-5, and 47% identity to caspase-1. Based on the x-ray crystal structure of ICE (29), several amino acid residues critical for binding and catalysis have been identified. These residues include the catalytic dyad Cys325 and His327, and Gly328, which stabilizes the tetrahedral intermediate. Arg179, Gln283, Arg341, and Ser347 form the binding pocket for the S1 subsite. These seven residues are conserved in all caspases thus far characterized including ERICE (Fig. 1B). Although the sequence homology is very high between ERICE and caspases-4 and -5, residues that make up the P1-P1′ binding pocket are not conserved, suggesting a different substrate specificity. The QAC(R/Q/G)G motif conserved in all caspases is conserved in ERICE (Fig. 1B). ERICE is the 13th member of the caspase family (Fig. 1C) and is tentatively designated caspase-13.

Tissue Distribution of ERICE—Northern blot analysis revealed ERICE to be constitutively expressed mainly in peripheral blood lymphocytes, spleen, and placenta. The transcript size was 1.6 kilobases corresponded to the full-length cDNA obtained from the fibroblast cDNA library (Fig. 2A). ERICE was highly expressed in HeLa cells but not in transformed hematopoietic cell lines including Burkitt’s lymphoma, Raji cells, or in promyelocytic leukemia HL-60 cells (Fig. 2B).

Overexpression of ERICE Induces Apoptosis—To study the functional role of ERICE, we transiently transfected MCF7 breast carcinoma cells and 293 embryonic kidney cells with an...
expression vector encoding full-length ERICE and assayed for apoptosis. Like other caspases, ERICE was able to induce cell death (Fig. 3, A and B). However, unlike caspase-4 and caspase-5, removal of the prodomain was not necessary to induce apoptosis (12). ERICE-transfected MCF7 and 293 cells displayed morphologic alterations typical of adherent cell lines undergoing apoptosis (Fig. 3C). To determine whether protease activity was required for inducing apoptosis, the catalytic cysteine was altered to an alanine. Consistent with a requirement for enzymatic activity, the Cys to Ala mutant did not induce apoptosis.
apoptosis in either the MCF7 or 293 cells. Furthermore, apoptosis induced by ERICE was efficiently blocked by virally encoded caspase inhibitors p35 and CrmA (Fig. 3, A and B).

**Activation of ERICE**—Although overexpressed ERICE induces apoptosis, this may not reflect its primary function because other ICE subfamily members do not appear to play a
prominent proapoptotic role. In keeping with this, granzyme B, which is known to induce apoptosis through the productive processing of caspases, cleaves but does not activate caspase-1, the prototypical member of the ICE subfamily. Additionally, members of the ICE subfamily are not productively processed by caspase-8, the apical caspase involved in proximal death receptor signaling. To address whether ERICE was productively processed by granzyme B or caspase-8, it was incubated with either protease (Fig. 4, A and C), and the emergence of active ERICE was assessed by reaction with biotinylated YVAD chloromethyl ketone, which covalently binds the catalytic cysteine within the large subunit of proteolytically competent (active) caspases (Fig. 4B). Caspase-8 processing of ERICE led to the generation of two subunits. One of the subunits was the prodomain plus the large catalytic subunit (pro1large), and the other was the small catalytic subunit. This is similar to the activation of caspase-1 in which the p45 zymogen must initially be processed to a stable active p35 pro1large subunit. Further processing, namely cleavage between the pro and large subunit, is highly dilutional sensitive and very inefficient in vitro such that in vitro translated zymogens do not undergo complete processing. Given the low concentration of in vitro translated ERICE, it was not surprising that caspase-8 processed it only to the pro+large and small subunits. Regardless, caspase-8-processed ERICE was efficiently labeled with biotinylated YVAD, indicative of generation of active ERICE (Fig. 4B). Granzyme B appeared to be more promiscuous, cleaving ERICE into a number of discrete fragments (Fig. 4C), none of which labeled with biotinylated YVAD (data not shown). In vivo, mutant ERICE was processed by both caspase-8 and Fas in a CrmA-inhibitable fashion (Fig. 5). Therefore, although ERICE is cleaved by both caspase-8 and granzyme B, it is only cleavage by caspase-8 which is productive and leads to the generation of active ERICE.

To map caspase-8 processing sites within ERICE, potential aspartate cleavage sites were mutated and tested as caspase-8 substrates. Productive cleavage was found to require Asp289 as alteration of this residue abolished processing (Fig. 4D). Therefore, ERICE must be cleaved by caspase-8 following Asp289 to yield an active heterodimeric enzyme. Notably, this aspartate is found in the sequence context LEED (residues 286–289) which is the preferred substrate for caspase-8 cleavage. In summary, this is the first example of an ICE subfamily member
that is activated by caspase-8 and suggests a potential downstream role for active ERICE in caspase-8-mediated cell death.

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