I-FLICE, a Novel Inhibitor of Tumor Necrosis Factor Receptor-1- and CD-95-induced Apoptosis

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The pivotal discovery that the death proteases caspase 8 (FLICE) and caspase 10 (Mch4/FLICE2) are recruited to the CD-95 and tumor necrosis factor receptor-1 signaling complexes suggested a mechanism by which these cytotoxic receptors to initiate apoptosis. In this report, we describe the cloning and characterization of I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD-95-induced apoptosis. The overall architecture of I-FLICE is strikingly similar to that of FLICE and Mch4/FLICE2. However, I-FLICE lacks both a catalytic active site and residues that form the substrate binding pocket, in keeping with its dominant negative inhibitory function. I-FLICE is the first example of a catalytically inert caspase that can inhibit apoptosis.

The cell death machinery is conserved throughout evolution and is composed of activators, inhibitors, and effectors (1). The effector arm of the cell death pathway is composed of a rapidly growing family of cysteine aspartate-specific proteases termed caspases (2). As implied by the name, these cysteine proteases cleave substrates following an aspartate residue (2, 3). Caspases are normally present as single polypeptide zymogens that bind to either FADD (MC159) or FLICE (E8) and disrupt assembly of the receptor signaling complex. Taking these data together, it can act as a dominant negative inhibitor of apoptosis.

MATERIALS AND METHODS

Cell Lines and Expression Vectors—Human embryonic kidney 293, 293T, and 293-EBNA cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, nonessential amino acids, t-glutamine, and penicillin/streptomycin. Expression constructs were made in pcDNA3 or pcDNA3.1/mycHisA (Invitrogen) using standard recombinant methodologies (20).

Cloning of I-FLICE—cDNAs corresponding to the partial open reading frame of I-FLICE were identified as sequences homologous to FLICE and Mch4/FLICE2 on searching the Human Genome Sciences data base using established expressed sequence tag methods (21, 22). Full-length cDNAs were obtained by screening a random-primed human umbilical vein endothelial cell cDNA library constructed in the pcDNA1 vector (Invitrogen). The sequence of I-FLICE was confirmed by sequencing plasmid DNA template on both strands by the dyeoxy chain termination method employing modified T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.)

Northern Blotting—Human multiple tissue and human cancer cell line poly(A)+ RNA blots were obtained from CLONTECH and processed according to the manufacturer’s instructions.

Transfection, Immunoprecipitation, and Western Analysis—Transient transfections of 293T cells were performed as described previously (23). Cells were harvested 48 h following transfection, immunoprecipitated with the indicated antibodies, and analyzed by immunoblotting.

Cell Death Assay—Human embryonic kidney 293 (for TNFR-1 killing) or 293 EBNA cells (for CD-95 killing) were transiently transfected with the indicated antibodies, and analyzed by immunoblotting.
cells. All assays were evaluated in duplicate, and the mean and standard deviations were calculated.

RESULTS AND DISCUSSION

Sequence of I-FLICE—Sequence analysis of a full-length cDNA revealed a 1443-base pair open reading frame that encoded a novel protein with a predicted molecular mass of 55.3 kDa (Fig. 1A). The pentapeptide sequence of I-FLICE, the pentapeptide corresponding to the conserved active site (QACR(Q)G) of other caspases is boxed. Shown underneath are schematic models of I-FLICE, FLICE, and Mch4. The N-terminal portion of each molecule contains two DED-like domains (shaded boxes), and the C-terminal portion contains both large and small catalytic subunits (p20/p10). The pentapeptide QNYVV of I-FLICE and the corresponding motifs in FLICE and Mch4 are indicated. B, the two DED motifs of I-FLICE were aligned with DED motifs present in FADD, FLICE, and Mch4. C, the C-terminal region of I-FLICE was aligned with the catalytic domains of FLICE and Mch4. ● symbols indicate residues involved in catalysis, and ▲ symbols identify residues that form the binding pocket for the carboxylate side chain of P1 Asp.

FIG. 1. Sequence analysis of I-FLICE. A, deduced amino acid sequence of I-FLICE. The pentapeptide corresponding to the conserved active site (QACR(Q)G) of other caspases is boxed. Shown underneath are schematic models of I-FLICE, FLICE, and Mch4. The N-terminal portion of each molecule contains two DED-like domains (shaded boxes), and the C-terminal portion contains both large and small catalytic subunits (p20/p10). The pentapeptide QNYVV of I-FLICE and the corresponding motifs in FLICE and Mch4 are indicated. B, the two DED motifs of I-FLICE were aligned with DED motifs present in FADD, FLICE, and Mch4. C, the C-terminal region of I-FLICE was aligned with the catalytic domains of FLICE and Mch4. ● symbols indicate residues involved in catalysis, and ▲ symbols identify residues that form the binding pocket for the carboxylate side chain of P1 Asp.

FIG. 2. Tissue distribution of I-FLICE. Human adult and cell lines poly(A)1 Northern blots were probed with 32P-labeled human I-FLICE probe corresponding to codons 215–331. PBL, peripheral blood leukocyte. The mouse tissue was Northern probed with a 200-base pair mouse I-FLICE probe complementary to the 5′ end.

and 18% identity (32 and 28% similarity) to FLICE and Mch4, respectively. Importantly, I-FLICE did not contain the catalytic cysteine that is normally embedded in the conserved pentapeptide QACRG or QACQG motif present in all known caspases. Rather, the pentapeptide sequence was QNYVV. In addition, based on the x-ray crystal structure of caspase-1 (and caspase-3), amino acid residues His237 (His121), Gly238 (Gly122), and Cys285 (Cys163) are involved in catalysis, whereas residues Arg179 (Arg64), Gln283 (Gln161), Arg341 (Arg207), and Ser347 (Ser213) form a binding pocket for the carboxylate side chain of the P1 aspartic acid (4–6). These seven residues are conserved in all caspases, but only three of them (Gly, Gln, and Ser as indicated in Fig. 1C) are found in I-FLICE. Given this lack of conservation of key residues involved in catalysis and substrate
I-FLICE binds FLICE and FLICE2 but not FADD. 293T cells were co-transfected with indicated plasmids. Following transfection, cells lysates were immunoprecipitated (IP) and immunoblotted with the respective epitope tag antibodies. W. Blot, Western blot.

Inhibitor of TNFR-1- and CD-95-induced Apoptosis

Overexpression of I-FLICE attenuated TNFR-1-induced (A) and CD-95-induced (B) cell death. 293 (A) or 293-EBNA (B) cells were co-transfected with the indicated plasmids together with the reporter construct pCMV β-galactosidase. The data shown are the percentages of blebbing blue cells as a function of total number of blue cells counted.

I-FLICE associated with FLICE and FLICE2—Previous studies have shown that the DED domain is a protein interaction motif that mediates the binding of the adaptor molecule FADD to the effector protease FLICE and Mch4/FLICE2 (8, 10). Given the striking structural similarity, we asked if I-FLICE interacted with either FADD or other FLICE-like caspases. Co-immunoprecipitation analysis clearly revealed the ability of I-FLICE to bind FLICE and Mch4/FLICE2 (Fig. 3A), but not FADD (Fig 3B). In this respect, I-FLICE resembles the viral DED-containing molecule E8, in that it binds FLICE but not FADD (17, 18). Because there was no association between I-FLICE and FADD, I-FLICE was not recruited to the CD-95 or TNF-1 signaling complex as evidenced by its inability to co-purify with these receptors (data not shown).

I-FLICE Inhibits TNFR-1 and CD-95-induced Apoptosis—Given the ability of catalytically inactive I-FLICE to complex with FLICE-like caspases, we reasoned that I-FLICE may be acting as a dominant negative inhibitor because the active form of all caspases is a tetramer derived from the processing of two zymogen forms to a four-chain assembly (4–6). It follows that a catalytically inert zymogen, such as I-FLICE, would be processed to inactive subunits that would result in the generation of a nonfunctional tetrameric protease. Although it is presently conjecture, this putative mechanism does predict that I-FLICE should inhibit TNFR-1 and CD-95-induced apoptosis where FLICE-like caspases play an initiating role. Consistent with the prediction, overexpression of I-FLICE resulted in substantial inhibition of TNFR-1-induced cell death comparable with previously characterized inhibitors including CrmA, MCl59, dominant negative FLICE, and dominant negative Mch4/FLICE2 (Fig. 4A). However, under the present experimental conditions, I-FLICE appeared to be a less potent inhibitor of CD-95-induced cell death, possibly reflecting the more potent death signal that emanates from this receptor.

In summary, our studies have identified a catalytically inactive member of the caspase family that can serve as a dominant negative inhibitor of CD-95- and TNFRI-induced cell death by binding and antagonizing the apical FLICE-like caspases. Additional studies will be necessary to work out in detail the exact nature of the inhibitory mechanism.

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