Membrane Oligomerization and Cleavage Activates the Caspase-8 (FLICE/MACHα1) Death Signal*

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Many forms of apoptosis, including that caused by the death receptor CD95/Fas/APO-1, depend on the activation of caspases, which are proteases that cleave specific intracellular proteins to cause orderly cellular disintegration. The requirements for activating these crucial enzymatic mediators of death are not well understood. Using molecular chimeras with either CD8 or Tac, we find that oligomerization at the cell membrane powerfully induces caspase-8 autoactivation and apoptosis. Death induction was abrogated by the z-VAD-fmk, z-IETD-fmk, or p35 enzyme inhibitors or by a mutation in the active site cysteine but was surprisingly unaffected by death inhibitor Bcl-2. Amino acid substitutions that prevent the proteolytic separation of the caspase from its membrane-associated domain completely blocked apoptosis. Thus, oligomerization at the membrane is sufficient for caspase-8 autoactivation, but apoptosis could involve a death signal conveyed by the proteolytic release of the enzyme into the cytoplasm.

A pivotal biochemical event of programmed cell death or apoptosis is the activation of cysteinyl, aspartate-specific proteases or caspases (1, 2). The caspase gene family in mammals includes at least 10 members that share protein sequence similarity to the ced3 cell death gene from Caenorhabditis elegans (3). The participation of caspases in programmed cell death is conserved widely in phylogeny from the nematode, C. elegans to humans (4). The essential role of caspases is to endoproteolytically cleave a select group of cellular proteins at aspartate residues, thereby causing the nuclear and cytoplasmic alterations that typify apoptosis. The principal regulation of caspases is post-translational. They reside in the cell as inactive zymogens, which must be proteolytically processed at internal aspartates to generate the subunits of the active enzyme.

How caspases are activated is a critical question in the immune system, since normal lymphocyte homeostasis and immune tolerance involves CD95-induced apoptosis that depends on caspase activation (5). Caspase activation is defective in patients that have inherited mutations in CD95 and suffer from the autoimmune/lymphoproliferative syndrome (ALPS).1, 2 (6, 7). The activation of caspase-8 (FLICE/MACH) appears to be the first step in the cascade of apoptotic events induced by CD95 (5, 8). Caspase-8 is recruited to the “death-inducing signal complex” (DISC), a multiprotein complex that forms rapidly on the cytoplasmic portion of the Fas/APO-1/CD95 receptor after ligand engagement, by the adapter protein FADD/MORT1 (8–15). The caspase-8 precursor protein is cleaved at 3 aspartate residues to become active, but processing has only been demonstrated by exposing the caspase-8 precursor to an active DISC complex, raising the important question of how activation is initiated (12). We therefore investigated the requirements of caspase-8 activation and apoptosis induction.

**EXPERIMENTAL PROCEDURES**

*Materials*

The PCR3-uni vector and TA cloning kit were from Invitrogen, San Diego, CA. The vectors pCEFL, pCEFL-caspase-8, and pCEFL-Myr containing the src myristoylation sequence were gifts from Dr. J. Silvio Gutkind, NIDR, NIH. The FADD-AU-1 pcDNA3 was provided by Dr. Vishva Dixit (13), and p35-pc1 and 3LacZ plasmids were provided by Dr. John Bertin, NIAID, NIH. The Taq and Pfu polymerases and the rapid DNA ligation kit were from Boehringer Mannheim. z-VAD-fmk (N-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) was from Enzyme Systems Products. PE-labeled anti-human CD8 and anti-Tac antibodies were obtained from Pharmingen, San Diego, CA, anti-GFP mAb was from CLONTECH, and horseradish peroxidase-conjugated goat-anti-mouse IgG was from Jackson ImmunoResearch. SuperSignal horseradish peroxidase substrate was from Pierce. The parental and Bcl-2-overexpressing stable MCP-7 lines were kindly provided by Drs. Ulrich Brinkmann and Ira Pastan, National Cancer Institute, NIH.

Methods

**DNA Constructions**—The caspase-8 (MACH-α1/FLICE) coding sequence was cloned using reverse transcription PCR into the pCR3-uni vector using the TA cloning kit per manufacturer’s protocols. The full-length cDNA was sequenced and then subcloned into a modified pCDNA3 vector, pCEFL, in which the cytomembranovirus promoter was replaced by the promoter for elongation factor 2 (EF-2). High-fidelity PCR products of caspase-8 (98–479 and 209–479) were subcloned as HindIII–NotI fragments into digested pCEFL. The vector pCEFL-C8-EMPTY vector and the pCEFL-Myr vector containing the src myristoylation sequence were used for the in-frame cloning of the caspase-8 protease domain. The Tac-C construct was made by amplifying the extracellular and transmembrane domains (Tac EX-TM) of the Tac cDNA using high-fidelity PCR. CD8 was removed from the CD8-C construct using HindIII and BamHI, and the digested PCR product of Tac EX-TM was ligated in frame with caspase-8 2209.

**Site-directed Mutagenesis**—Point mutations were made in the pCEFL-C8 vector using the altered sites mutagenesis kit (Promega, Madison, WI) and the Quik-Change kits (Stratagene), according to the instructions of the manufacturers.

**Cell Death Assays**—For all Jurkat transfections, plasmid constructions (pCEFL, pCEFL-caspase-8, pCEFL-caspase-8 Δ98, pCEFL-caspase-8 2209, pCEFL-Myr-C, pCEFL-caspase-8, pCEFL-Tac-C, and pDNA3-FADD, p35-pc1, CD8, C, and the Tac-C mutants as outlined in the figures), along with pCEFL-GFP, were electroporated into 4–8 × 10⁶ Jurkat cells in 0.4 ml of complete medium in an Electrocell Manipulator.
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600 (BTX Corp., San Diego, CA). Plasmids were added to the cells at a 3:1 mass excess of the caspase constructions to GFP to ensure that all cells expressing GFP simultaneously expressed the cotransfected DNA of interest. The total amount of DNA/cuvette ranged from 15 to 20 µg. After pulse discharge at settings 260 V, 1050 µF, and 7200, the cells were immediately placed in 7 ml of fresh RPMI medium containing 10% fetal calf serum, incubated for 16–20 h at 37 °C, and analyzed using flow cytometry. Where indicated 50 µM of the caspase inhibitor z-VAD-fmk was added to the cells/media immediately following electroporation. For the chimeric caspase-8 constructs, the cells were stained for surface expression of either CD8 or Tac (CD25) prior to analysis using flow cytometry. Dead cells were gated out using forward and side scatter profiles, and the percent cell death was calculated from the loss of live GFP-positive cells in treated samples compared with the control vector. For the inhibition of CD8-C apoptosis in Jurkat T-cells, 4 µg of CD8-C (or pCEFL control plasmid) was cotransfected with 2 µg of the GFP vector and either 20 µg of pCEFL with or without 50 µM z-VAD-fmk, 50 µM z-IETD-fmk (Enzyme Systems Products, Livermore, CA) or 20 µg of pS8-pC1. After transfection, the cells were analyzed for CD8 surface expression using flow cytometry.

For 293T cells, subconfluent cells were transfected in six-well plates using the calcium phosphate method (Stratagene) according to manufacturer’s instructions, with the modification that 25 µM chloroquine was added to the medium to facilitate DNA uptake. Cells were transfected with the plasmid combinations described in the figures with a 2.5:1 DNA excess of the DNA of interest to the 3 Lac-z construct (total DNA: 1.5–2.0 µg). After 24–30 h, the cells were fixed in 2% formaldehyde, 0.2% glutaraldehyde in phosphate-buffered saline for 15 min at room temperature and then stained in phosphate-buffered saline containing 5 mM each KFe(CN)₆ and K₄Fe(CN)₆, 3 mM MnCl₂, 2 mM MgCl₂, 0.02% SDS, 0.02% Nonidet P-40, and 1 mg/ml 5-bromo-3-chloro-3-indolyl-p-d-galactopyranoside until a suitable color developed, usually for 3–4 h at 37 °C. To enumerate the fraction of blue cells that had undergone apoptotic changes, a minimum of 200 blue-staining cells/well were counted using light microscopy and unambiguously scored as being apoptotic or non-apoptotic by morphological assessment. The Bel-2 stable MCF-7 line showed approximately 20-fold excess Bel-2 protein over the parental line by Western blot and had a demonstrable defect in tumor necrosis factor-induced apoptosis (data not shown). Transfections were done using the calcium phosphate method as described above. For the CD8-C inhibition experiments in 293T cells, 250 ng of CD8-C was transfected along with 1–5 µg of either CD8-empty or CD8-C D210A/D216A or 5 µg of pCEFL using Superfect (Qiagen) according to manufacturer’s protocol. Cells were fixed at 24 h, stained, and enumerated with light microscopy as described above.

Flow Cytometry Analysis—Surface expression of Jurkat T-cells transfected with CD8 and Tac fusion proteins was done using PE-labeled anti-human CD8 and anti-Tac antisera (Pharmingen, San Diego, CA). Flow cytometry was carried out on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) using CellQuest software. GFP fluorescence was analyzed using the FL1 channel.

Western Blotting—Twenty-four hours after transfection (as described above) with the indicated constructs (see figure legends), 293T cells were lysed in buffer containing 140 mM NaCl, 10 mM Tris (pH 7.2), 2 mM EDTA, 1% Nonidet P-40, complete protease inhibitor mix (Boehringer Mannheim), and 10 mM iodoacetamide. After lysis for 30 min on ice, supernatants were diluted in SDS sample buffer with or without 40 mM dithiothreitol, boiled, and electrophoresed on 4–20% Tris/glycine/SDS gels. Proteins were blotted onto nitrocellulose using a semidy transfer apparatus. The blots were then probed with 1:1000 dilution of anti-GFP mAb (Fig. 3) followed by 1:10,000 dilution of goat anti-mouse horseradish peroxidase (Jackson Immunoresearch). For the inhibition experiments in 293T cells, 250 ng of CD8-C was transfected along with 1–5 µg of either CD8-empty or CD8-C D210A/D216A or 5 µg of pCEFL using Superfect (Qiagen) according to manufacturer’s protocol. Cells were fixed at 24 h, stained, and enumerated with light microscopy as described above.

RESULTS AND DISCUSSION

We first studied caspase-8 by transfecting expression constructs containing either full-length or truncated versions into Jurkat T-cells (Fig. 1). We found that the full-length protein inefficiently induced apoptosis compared with the death-signaling protein, FADD/MORT1 (10, 13) (Fig. 2A). Removal of one or both death effector domains (DEDs) decreased rather than increased apoptosis demonstrating that the caspase domain does not inhibit formation of the active protease. Protein blots confirmed that equivalent protein expression was obtained with each of the constructs (data not shown). Thus, simple overexpression of this caspase did not efficiently induce activation or apoptosis in Jurkat T-cells. We therefore tested the concept that membrane localization and/or oligomerization could initiate caspase autoactivation and apoptosis induction as might be envisioned based on the observation that caspase-8 can be recruited to the DISC (8–11). The caspase domain contains two subunits, p18 and p11, generated by proteolytic processing at aspartate residues (D) as shown. The active site cysteine at amino acid 360 (C360) is indicated. Caspase-8 AΔ98 and Δ209 lack the first 97 and 208 amino acids, respectively; the first five amino acids at the N termini are shown. Myristoylated-caspase (Myr-C) consists of the myristoylation sequence from the src gene (17) attached to the N terminus of caspase-8 Δ209. Tac-C and CD8-C consist of the extracellular (patterned) and transmembrane (solid) domains from Tac (CD25/IL-2R) and CD8α, respectively, fused in frame with caspase-8 Δ209. CD8-empty is the same construction as CD8-C except that its cytoplasmic domain is truncated at the caspase-8 fusion point.
prepared a chimera, Tac-C, between the interleukin-2 receptor α chain (Tac) extracellular and transmembrane domains and the caspase domain (18). Overexpression of Tac has been recently shown to cause self-association in the absence of ligand; that Tac-C, similar to CD8-C, greatly augmented apoptosis, even without antibody cross-linking of the Tac moiety (Fig. 2C), suggesting that spontaneous oligomerization of the extracellular domains of CD8 or Tac is sufficient to powerfully induce caspase-8 autoactivation and apoptosis.

The cell death caused by CD8-C involved caspase activation, because either the z-VAD-fmk peptide (19) or the baculovirus p35 caspase inhibitors (20) inhibited apoptosis (Fig. 2D). Under caspase-8-inhibited conditions, we observed abundant surface CD8 staining on viable cells, which confirmed that the CD8-C chimera was actually expressed following transfection (Fig. 2E), and found that this peptide completely abrogated apoptosis induced by CD8-C (Fig. 2F). The viral inhibitory protein MC159, which disrupts normal DISC formation and blocks CD95-induced apoptosis by preventing caspase-8 from binding to FADD (22, 23), also did...
not inhibit apoptosis by CD8-C (data not shown), implying that the autoactivation of caspase-8 in our system did not require the formation or participation of the DISC. Also, brefeldin A was incapable of blocking CD8-C-mediated death, implying that oligomerization and caspase activation may occur in the membranes of early compartments prior to transport to the membrane at the cell surface (data not shown).

An important biochemical feature of the CD8α extracellular domain is its ability to form disulfide-linked homodimers. Since no additional external cross-linking (such as by antibody) was required for the powerful apoptosis induction by CD8-C, we reasoned that dimerization was critical in promoting association and processing of the caspase precursors into an active form. To assess whether CD8-C had undergone dimerization, we analyzed detergent lysates from 293T cells that were transfected with a construct expressing a CD8-C linked to GFP (24) (CD8-C-GFP), either alone or together with constructs expressing CD8-empty or an inactive CD8-C without the GFP tag (CD8-C:D210A/D216A, see below). Western blots with an anti-GFP mAb showed that CD8-C-GFP formed an apparent dimer complex (molecular mass \(=170\) kDa, lane 4) in nonreducing conditions, but only monomers (molecular mass \(=85\) kDa) in reducing conditions (Fig. 3A). Coexpression of CD8-C-GFP and either an inactive CD8-C chain without the GFP tag or CD8-empty caused a decrease in the CD8-C-GFP homodimer and the appearance of apparent heterodimer complexes (molecular mass \(=142\) kDa, lanes 1, 2, and 5), which was absent without CD8-C-GFP. Similar results were obtained with coexpression of CD8-C-GFP and CD8-empty, with smaller heterodimeric complexes. Thus, consistent with the formation of disulfide-linked homodimers by native CD8α (15, 16), the CD8-C chimera formed dimers with itself and other CD8α expression proteins. To determine if dimerization was essential for caspase activation, we tested whether coexpression of CD8-empty or an inactive CD8-C chimera (D210A/D216A, see below) dominantly interfered with the ability of CD8-C to induce apoptosis. Cotransfection of these constructs confirmed this prediction (Fig. 3B). We found that either CD8-empty or an inactive CD8-C chimera blocked the lethality of CD8-C in a dose-response fashion (Fig. 3B).

We next investigated how enzymatic processing of CD8-C could lead to apoptosis. With a single nucleotide change, we substituted serine for the catalytic site cysteine (CD8-C:C360S). This change completely abrogated apoptosis, indicating that the active site cysteine was indispensable (Fig. 4A). We therefore assessed the functional importance of aspartate residues that reside at the site for cleaving the prodomain from the caspase domain (which have been preserved in both the CD8-C and Tac-C constructs) by mutations. We found that the substitution of alanines for the Asp\(^{210}\) and Asp\(^{216}\) residues unexpectedly blocked apoptosis by CD8-C (Figs. 1 and 4C). The two aspartates were not equivalently important, since the D216A mutation only modestly reduced apoptosis, whereas mutation of Asp\(^{210}\) or both Asp\(^{210}\) and Asp\(^{216}\) completely inhibited apoptosis (Fig. 4B). This striking effect was also observed with the corresponding mutations in the Tac-C chimera (Fig. 4C and data not shown). Additionally we performed a Western blot of 293T cells transiently transfected with either CD8-C or the double mutant CD8-C D210/216A using a mAb specific for caspase-8 to examine processing of the caspase chimera. The wild-type CD8-C underwent cleavage into processed fragments, which were released into the soluble cytosolic fraction, whereas the double mutant chimera did not (Fig. 4D). No cleavage products were found in the membrane-associated pellet for either the wild-type or mutant molecules (data not shown). Thus, even with an intact catalytic site cysteine, the lethality of the CD8-C or Tac-C chimeras is lost if the caspase cannot be proteolytically cleaved at the point of its association with the membrane.

Caspase activation is a crucial biochemical event involved in
most, if not all, forms of apoptosis, so it is of central importance to understand the activation requirements of these enzymes (1, 2). Our results show that membrane-associated oligomerization of caspase-8, the most proximal caspase in the CD95 signal cascade, is sufficient to powerfully induce apoptosis in several different cell types. Although previous studies have detected the cleaved caspase-8 prodomain in the “DISC” proteins aggregated with the cytoplasmic tail of CD95, these studies were limited by the fact that they did not determine the stoichiometry of the proteins to know if oligomerization of multiple caspase-8 molecules was likely to have occurred (8–11). Furthermore, the previous two-dimensional gel analyses were descriptive and did not directly test the necessity of cleavage at various aspartate residues for apoptosis induction. Our data address these issues and suggest that there are two critical steps in the caspase-8 death pathway. First, we have shown that oligomerization is sufficient to activate the enzyme. Homodimers of the wild-type CD8-caspase induced death, whereas heterodimers between the wild-type and an inactive CD8 construct were unable to stimulate death. Second, we have shown that proteolytic cleavage of the active caspase at the point of its membrane association was also required. Death induced by the caspase chimera occurred spontaneously without cross-linking of the CD8 extracellular domain. Also our results suggest that apart from the ability of CD95 and FADD/MORT1 to bring the caspase-8 molecules together, the DISC complex is not required for autocatalytic activation. Thus our data suggest that dimerization of caspases favors the spontaneous generation of an unstable, but active, conformation that can initiate autoprocessing into a thermodynamically more stable caspase. Although our experiments demonstrate that membrane-linked oligomerization causes activation, it is very likely that oligomerization of the enzyme within the cytosol may also strongly activate caspase activity. The crystal structures of active caspase tetramers show that each partner in a pair of large or small subunit chains is positioned antiparallel with respect to the other (25, 26). Since our chimeras constrain the N termini of the precursors in a parallel relationship, the pair of large or small subunit chains is positioned antiparallel with respect to the other (25, 26). Since our chimeras constrain the N termini of the precursors in a parallel relationship, the active caspase from membrane association is important for apoptosis. Detachment of the caspase from the membrane may release the mature enzyme into the cytoplasm where it may catalyze apoptosis substrates that could be sequestered away from the cell membrane. Alternatively, cleavage away from the prodomain may also increase the enzymatic activity or stability; however, enzymatic activity has been found to be associated with the DISC complex (12). Unlike other membrane signaling pathways such as phosphorylation or inositol phosphate generation, caspase-8 processing is an irreversible signal that commits the cell’s fate to apoptosis. These downstream biochemical events involving caspase-8 may be targets for genetic alterations in autoimmune/lymphoproliferative syndrome patients who do not have mutations in the CD95 protein itself (32).

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