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Activation of Caspase-2 in Apoptosis*

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Members of the CED-3/interleukin-1 β -converting enzyme (ICE) protease (caspase) family are synthesized as proforms, which are proteolytically cleaved and activated during apoptosis. We report here that caspase-2 (ICH-1/NEDD-2), a member of the ICE family, is activated during apoptosis by another ICE member, a caspase-3 (CPP32)-like protease(s). When cells are induced to undergo apoptosis, endogenous caspase-2 is first cleaved into three fragments of 32–33 kDa and 14 kDa, which are then further processed into 18- and 12-kDa active subunits. Up to 50 μ M N-acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO), a caspase-3-preferred peptide inhibitor, inhibits caspase-2 activation and DNA fragmentation *in vivo*, but does not prevent loss of mitochondrial function, while higher concentrations of DEVD-CHO (>50 μ M) inhibit both. In comparison, although the activity of caspase-3 is very sensitive to the inhibition of DEVD-CHO (<50 nM), inhibition of caspase-3 activation as marked by processing of the proform requires more than 100 μ M DEVD-CHO. Our results suggest that the first cleavage of caspase-2 is accomplished by a caspase-3-like activity, and other ICE-like proteases less sensitive to DEVD-CHO may be responsible for activation of caspase-3 and loss of mitochondrial function.

Interleukin-1 β -converting enzyme (ICE)¹ caspase-1 (1, 2) was identified as the first mammalian homolog of the *Caenorhabditis elegans* cell death gene product CED-3 (3, 4). Subsequently, a growing number of ICE-like cysteine proteases have

been isolated and characterized, including caspase-2 (NEDD-2/ICH-1) (5, 6), caspase-3 (CPP32/YAMA/Apopain) (7, 8, 39), caspase-6 (Mch-2) (9), caspase-4 (TX/Ich-2/ICE_{rel}II) (10–12), caspase-5 (ICE_{rel}III) (12), caspase-7 (Mch-3/CMH-1/ICE-LAP3) (13–15), caspase-8 (FLICE/MACH/Mch-5) (16–18), caspase-10 (Mch-4) (18), and caspase-9 (ICE-LAP6/Mch-6) (19, 20). Increasing evidence suggests that caspases play critical roles in the control of programmed cell death (for review, see Refs. 21–23). Microinjection of an expression vector encoding CrmA, a serpin encoded by cowpox virus, inhibits the death of dorsal root ganglia neurons induced by nerve growth factor deprivation (24). Viral inhibitors of caspases, p35 and CrmA, inhibit serum withdrawal-, tumor necrosis factor-, and Fas-induced apoptosis, as well as cytotoxic T lymphocyte (CTL)-mediated apoptosis (6, 25–29). *Ice*^{−/−} thymocytes undergo apoptosis normally when treated with dexamethasone and γ -irradiation but are partially resistant to Fas-induced apoptosis (30). Peptide inhibitors of caspases prevent programmed cell death when administered to tissue culture cells and animals (31). These results indicate that the ICE family plays important roles in mammalian apoptosis. The roles played by individual members of the caspase family in controlling apoptosis are the subjects of intensive debates and investigations.

Nedd-2, the murine caspase-2, was identified by Kumar *et al.* (32) as a mRNA expressed mostly during early embryonic brain development and down-regulated in adult brain. Overexpression of *Nedd-2* in cultured fibroblast and neuroblastoma cells results in cell death by apoptosis, which is suppressed by the expression of the human *bcl-2* gene (5). Previous work in our lab has shown that the human caspase-2, *Ich-1* (*Ice* and *ced-3* homolog), encodes a protein that shares sequence similarities with ICE and CED-3 proteins (6). Two different forms of mRNA species derived from alternative splicing encode two proteins, ICH-1_L and ICH-1_S, which have antagonistic effects on cell death. ICH-1_L (435 amino acids) contains sequence homologous to both p20 and p10 subunits of ICE, while ICH-1_S (312 amino acids) is a truncated version of ICH-1_L, containing only the p20 region. Previous studies of *Ich-1* in our laboratory revealed that overexpression of *Ich-1*_L induces programmed cell death, while overexpression of *Ich-1*_S suppresses Rat-1 cell death induced by serum deprivation. These results suggest that *Ich-1* may play an important role in both positive and negative regulation of programmed cell death. Apoptosis induced by ICH-1 is suppressed by overexpression of *bcl-2*, but not by *crmA*. Northern blotting and reverse transcription-PCR results showed that *Ich-1* is expressed in many tissues and cells with tissue and developmental stage specificities. Expression of *Ich-1* is detected in HeLa, THP.1, U937, and Jurkat cells. The expression patterns of these two alternatively spliced forms of *Ich-1* show tissue-specific differences; expression of both *Ich-1*_L and *Ich-1*_S can be detected in heart, kidney, and embryonic and adult

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¹ The abbreviations used are: ICE, interleukin-1 β -converting enzyme; CPP32, cysteine protease p32; CTL, cytotoxic T lymphocyte; DEVD-CHO, N-acetyl-Asp-Glu-Val-Asp-aldehyde; GB, granzyme B; ICH-1, ICE and CED-3 homolog 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; TNF, tumor necrosis factor; YVAD-CHO, N-acetyl-Tyr-Val-Ala-Asp-aldehyde; YVAD-CMK, N-acetyl-Tyr-Val-Ala-Asp-chloromethylketone; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; TBST, Tris-buffered saline with Tween 20.

brain with the expression of *Ich-1_S* being highest in embryonic brain, and only *Ich-1_L* is expressed in adult thymus.

To investigate the mechanism and function of caspase-2 (NEDD-2/ICH-1) in apoptosis, we examined the processing and activation of caspase-2 when cells undergo apoptosis. We demonstrate here that caspase-2 is processed and activated in a specific temporal sequence when cells are induced to undergo apoptosis by diverse stimuli. Our results show that caspase-2 is activated by a caspase-3 (CPP32)-like protease when cells are induced to undergo apoptosis. Moreover, caspase-2 activation can be distinguished from activation of caspase-3 and loss of mitochondrial function by their sensitivity to inhibitors of the ICE family.

MATERIALS AND METHODS

Reagents—Staurosporine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other molecular biology grade reagents were purchased from Sigma. *N*-Acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO), *N*-acetyl-Tyr-Val-Ala-Asp-chloromethylketone (YVAD-CMK), and *N*-acetyl-Tyr-Val-Ala-Asp-aldehyde (YVAD-CHO) were obtained from Bachem Bioscience, Inc. (King of Prussia, PA).

Cell Cultures—Jurkat cells were grown in RPMI 1640 medium (Life Technologies, Inc.) with 10% fetal calf serum. HeLa cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum.

Constructions of Expression Plasmids for Caspase-1, -2, and -3 in Bacteria and Site-directed Mutagenesis of Caspase-2—*Bam*HI sites were introduced at the 5' and 3' ends of the p30 domain of caspase-1 and full-length caspase-3 by PCR amplification. For caspase-1 p30, oligonucleotide primers 5'-CGCGGATCCTGGCACATTTCCAGGAC-3' (5' primer) and 5'-CGCGGATCCTAAGGAAGTATTGGC-3' (3' primer) were used. For caspase-3, two primers, 5'-CGCGGATCCTGGAGAACTGAAACTC-3' (5' primer) and 5'-CGCGGATCCTACCATCTTCTCACTTGG-3' (3' primer), were used. For caspase-3 p30, *Xho*I sites were introduced at the both ends by PCR using two primers: 5'-GCGCTCGAGGTCCTGTCTGCCT-3' (5' primer) and 5'-GCGCTCGAGGTGACATGTGGG-3' (3' primer). The PCR products were cloned into pBluescript (Promega, Madison, WI), and their sequences were confirmed by DNA sequencing (U. S. Biochemical Corp.). Each fragment was inserted into the *Bam*HI or *Xho*I site of pET-15b (Novagen, Madison, WI). The resulting plasmids were transformed into *Escherichia coli* strain BL21(DE3).

Site-directed mutagenesis of caspase-2 was carried out by PCR. Two primers, D316E primer (5'-GGGGATCCTGCGTGGTTCTTTCCCTCTTGTTGGTCT-3') and D330E primer (5'-GCAGGATCCCCCTGGGTGC-GAGGAGAGTGATGCCGGTAAAG-3') were used to mutate both Asp-316 and Asp-330 to Glu. To generate caspase-2 (D316E) mutant, PCR was performed using caspase-2 5' primer (5'-GCGCTCGAGCTGATGCGGCTG-3') and D316E primer and wild type caspase-2 cDNA as a template. The PCR fragment was cloned into pBluescript. The *Bam*HI-digested PCR fragment was used to replace the corresponding wild type fragment in caspase-2. D330E primer and caspase-2 3' primer (5'-CGGCTCGAGA CATCATGTGGG-3') were used in a similar procedure to generate caspase-2 (D330E) mutant.

Preparation of Bacterial Lysates Containing Caspase-1, -2, and -3 Activities—*E. coli* BL21(DE3) transformed with plasmids expressing caspase-1, -2, and -3 genes were grown in LB media to exponential phases, and induced with 0.4 mM isopropyl-1-thio- β -D-galactopyranoside for 2 h. Cells were pelleted, resuspended in lysis buffer (30 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 1% Nonidet P-40, and 20 μ g/ml PMSF), and sonicated. The supernatant after centrifugation at $14,000 \times g$ for 15 min was used in enzymatic cleavage assays. The protein concentration was determined by BCA assay (Pierce), and aliquots were stored at -80°C .

Determination of Cell Viability by Trypan Blue Exclusion and MTT Assays—Jurkat cells were induced to undergo apoptosis by a variety of agents including staurosporine and anti-Fas monoclonal antibody CH-11 (Kamiya Biomedical Co., Thousand Oaks, CA), whereas HeLa cells were treated with a combination of TNF α (R&D Systems, Minneapolis, MN) and cycloheximide. The percentage of cell death was measured either by trypan blue exclusion or MTT assays. For trypan blue exclusion assay, Jurkat cells or trypsinized HeLa cells were incubated with 0.4% trypan blue solution (Sigma) for 10 min, and more than 200 cells were scored on a hemocytometer. Alternatively, MTT assays were performed as described (33). Briefly, 5×10^4 cells (50 μ l) were subcultured in RPMI 1640 (phenol red-free) supplemented with 10% fetal calf

serum in a 96-well plate, and treated with apoptosis-inducing agents for various time periods. For MTT assay, 5 μ l of MTT agent (5 mg/ml in RPMI 1640 (phenol red-free)) was added and further incubated for 2 h. Equal volumes of 0.05 N HCl in isopropanol were then added, and cells were disrupted by pipetting up and down. Cell viabilities were determined colorimetrically by using an automated 96-well plate reader (Molecular Devices, Sunnyvale, CA) and SOFTmax software to measure absorbance at 570–650 nm.

DNA Fragmentation Assay—Detection of DNA fragmentation was performed as described by Eastman (34). Briefly, a 2% agarose gel was prepared by pouring 350 ml of 2% agarose in TAE buffer in a large (20 $\times$ 34 cm) horizontal gel support. Once the gel solidified, the top section of gel immediately above the comb was removed, and filled with 1% agarose, 2% SDS, 64 μ g/ml proteinase K. After treated with 200 ng/ml anti-Fas monoclonal antibody in the presence of different amounts of DEVD-CHO for 20 h, Jurkat cells were harvested by centrifugation at 1000 rpm, and excess medium was removed. The cell pellets were resuspended in 15 μ l of sample buffer (5% glycerol, 5 mM Tris, pH 8.0, 0.05% bromophenol blue, and 5 mg/ml RNase A), and directly loaded into the wells. After electrophoresis for 14 h at 60 V at room temperature, the gel was stained with 0.5 μ g/ml ethidium bromide in water for 1 h, and destained in water overnight. The picture was taken using the Gel Doc 1000 system (Bio-Rad).

Western Blotting—The protein samples were subjected to SDS-PAGE, and then transferred to Immobilon-P membranes (Millipore, Bedford, MA) using a semi-dry transfer apparatus (Pharmacia Biotech Inc.). The membranes were blocked in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween 20) containing 5% nonfat dried milk overnight at 4°C . Membranes were then blotted with various primary antibodies with different dilutions for 2 h at room temperature. After washing three times in TBST, membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (either goat anti-mouse or goat anti-rabbit) (Southern Biotechnology, Birmingham, AL) for 45 min. After washing in TBST, proteins were detected by ECL (Amersham) according to the manufacturer's instructions. Primary antibodies were diluted as follow: polyclonal antibody for caspase-2 with a dilution of 1:3000, polyclonal antibody C-20 (Santa Cruz) for caspase-2 C terminus (416–435 residues; SEYCSLTCRLYLFPGHPT) with a dilution of 1:200, monoclonal antibody for caspase-3 (Transduction Laboratories) with a dilution of 1:2000, polyclonal antibody for PARP with a dilution of 1:1000, and monoclonal antibody for α -tubulin (Sigma) with a dilution of 1:5000.

Preparation of Jurkat Cytosolic Lysates—Jurkat cells (1×10^8) were treated with 1 μ M staurosporine for various time periods, and cytosolic lysates were prepared as described with minor modification (35). Briefly, cells were washed twice with cold RPMI 1640, and resuspended in 400 μ l of extraction buffer (10 mM HEPES, pH 7.0, 40 mM glycerophosphate, 50 mM NaCl, 2 mM MgCl₂, 5 mM EGTA, and 1 mM DTT) containing protease inhibitors (1 mM PMSF, 1 μ g/ml leupeptin, 0.5 μ g/ml aprotinin). After four cycles of freezing and thawing, crude extracts were obtained by centrifugation at $12,000 \times g$ for 15 min at 4°C . The cell lysates were further centrifuged at $100,000 \times g$ for 60 min, and the resulting supernatant was used as the cytosolic fraction. The protein concentration was determined by BCA protein assay (Pierce), and aliquots were stored at -80°C .

In Vitro Cleavage Assays—*In vitro* translations of ^{35}S -labeled proteins were done by using the TNT-coupled transcription/translation kit (Promega) in the presence of [^{35}S]methionine. ^{35}S -Labeled proteins were incubated with either bacterial lysates or staurosporine-treated Jurkat cytosolic lysates in a reaction buffer (20 mM Tris-HCl, pH 7.5, 10 mM DTT, 0.1 mM EDTA) for 1–2 h at 30°C , in the presence of protease inhibitors (1 mM PMSF, 0.5 μ g/ml aprotinin). The reactions were terminated by addition of equal volume of 2 $\times$ protein lysis buffer, and analyzed by SDS-PAGE. In Granzyme B (GB) cleavage assay, ^{35}S -labeled caspase-2 or caspase-3 was incubated with 20 ng GB in a reaction buffer (20 mM Tris, pH 8.0, 100 mM NaCl, and 1 mM DTT) at 30°C for 1 h.

Analysis of Target Cell Proteins following Cytotoxic T Lymphocyte (CTL)-mediated Cytolysis—Alloreactive murine CTL (F3B4, anti-H-2^b, or FC4 and G4, anti-H-2^d) were harvested 4–6 days after stimulation and purified by Ficoll-Hypaque density gradient centrifugation. Target cells (EL-4, H-2^b; P815, H-2^d) were washed in fresh supplemented RPMI medium. Target cells ($\sim 6 \times 10^5$) were mixed with CTL at an effector:target ratio of 1–2.5 in a final volume of 200 μ l of medium in microcentrifuge tubes. Control samples were prepared by adding the cells directly to 1 ml of PBS wash buffer containing the protease inhibitors diisopropyl fluorophosphate (4 mM) and *para*-hydroxymercurobenzoate (2 mM) and immediately harvested. The remaining samples were

centrifuged briefly at 500 rpm, and then incubated at 37 °C for 45–90 min. The incubated samples were diluted with 1 ml of washing buffer and pelleted at 2000 rpm for 2 min in a microcentrifuge. The supernatants were aspirated, and the cell pellets were dissolved in 200 μ l of PBS solubilization buffer containing 1% Nonidet P-40, 0.1% SDS, diisopropyl fluorophosphate, and *para*-hydroxymercurobenzoate for 15 min on ice. The samples were centrifuged at 3000 rpm for 3 min in a microcentrifuge. The supernatants were transferred to fresh microcentrifuge tubes, and precipitated in 1.2 ml of cold acetone. After overnight storage at –20 °C, the extracted proteins were recovered by centrifugation, dried by vacuum centrifugation, and analyzed by SDS-PAGE.

RESULTS

Caspase-2 Is Processed and Activated during Apoptosis—Members of the caspase family are synthesized as precursors of approximately 45–50 kDa. Activation of the caspases involves proteolytic cleavages of the precursors at specific Asp residues into a large subunit of approximately 20 kDa and a small subunit of approximately 10 kDa. To determine whether caspase-2 is cleaved and activated when cells undergo apoptosis, a rabbit polyclonal antibody was generated against purified His-tagged caspase-2 protein expressed in *E. coli*. On Western blots, this antibody recognizes a 48-kDa polypeptide, the molecular mass predicted for caspase-2 precursor protein, in Jurkat and HeLa cells as well as non-human cell lines including Rat-1 and COS cells (Fig. 1 and data not shown). This 48-kDa protein is specifically absent from tissues of *caspase-2*^{–/–} mutant mice generated by gene targeting technique, which further confirms the identity of this 48-kDa protein as the product of caspase-2 locus (data not shown). In several human cell lines, as well as in mice, this polyclonal anti-caspase-2 antibody also detects a 37-kDa polypeptide, which is not altered in caspase-2^{–/–} mutant mice, and thus is not from caspase-2 locus (data not shown). No cross-reactivity of this antibody to caspase-1, caspase-3, and caspase-4 was observed using Western blot analysis (data not shown).

To examine whether caspase-2 is activated during apoptosis, we induced apoptosis in Jurkat cells by treatment with anti-Fas antibody in the presence or absence of cycloheximide, which inhibits protein synthesis and potentiates apoptosis, or staurosporine, a broad spectrum protein kinase inhibitor that induces apoptosis in a variety of cells (36). Total cell lysates were collected at different time points and subjected to Western blot analysis using the polyclonal anti-caspase-2 antibody. In these experiments, processing of pro-caspase-2 was first detected as the appearance of a 32–33-kDa doublet at 1-h time point and an 18-kDa peptide at 4-h time point (Fig. 1A). The degree of caspase-2 processing correlates very well with the extent of cell death. A similar processing pattern of caspase-2 was observed with anti-Fas antibody alone, but with a delayed time course of cell death and caspase-2 processing (data not shown). Processing of caspase-2 was also detected in HeLa cells that were induced to die by TNF α and cycloheximide (27) (Fig. 1B). These observations suggest that caspase-2 is activated in apoptosis and its processing may be an important regulatory step for caspase-2.

Processing of Pro-caspase-2 Occurs in Distinct Steps—As described above, a polyclonal anti-caspase-2 antibody first detects the appearance of 32–33-kDa doublets and then detects an 18-kDa polypeptide during the course of apoptosis. The 32–33-kDa doublets may be intermediate processing products, which may consist of either the large subunit plus pro-domain or the large subunit plus the small subunit. Since this polyclonal antibody against caspase-2 recognizes the purified full-length but not the small subunit of caspase-2 expressed in *E. coli* (data not shown), the 32–33-kDa products are likely to be the pro-domain plus the large subunit. To verify this, we used a polyclonal peptide antibody that recognizes the C terminus of

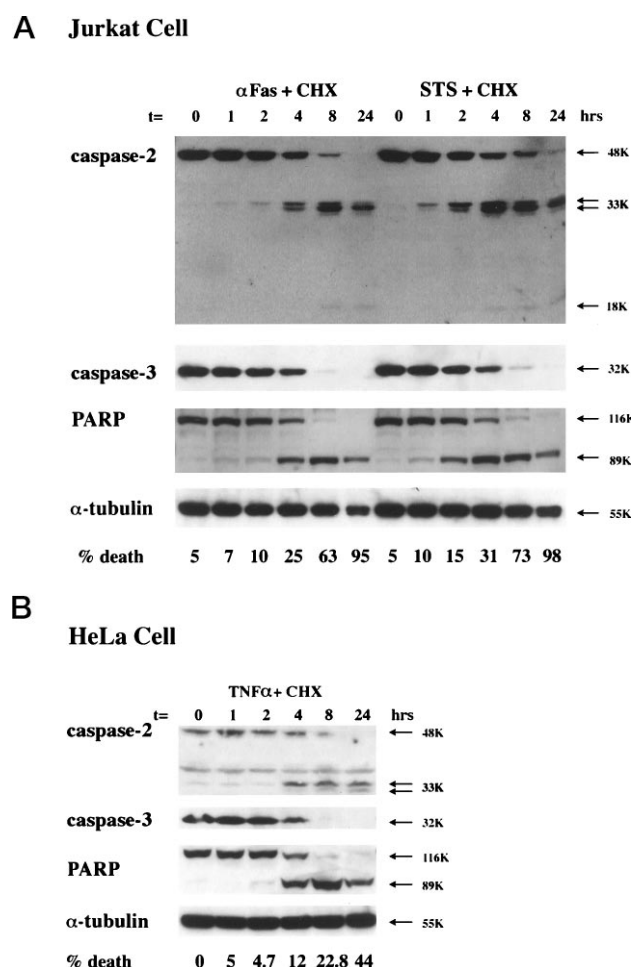


FIG. 1. The temporal profile of caspase-2 processing and activation during apoptosis. Individual caspase-2 fragments and their sizes are indicated. The percentages of cell death determined by trypan blue exclusion are indicated at the bottom. **A**, activation of caspase-2 and -3 and cleavage of PARP in apoptosis induced by anti-Fas antibody and staurosporine of Jurkat cells. 2×10^6 Jurkat cells were treated with either 10 ng/ml anti-Fas antibody (CH-11) or 0.1 μ M staurosporine (STS) in the presence of 50 μ g/ml cycloheximide (CHX) for various time periods as indicated. Aliquots of the total cell lysates were subjected to 13% SDS-PAGE, and immunoblotting was performed using various antibodies as indicated under "Materials and Methods," and proteins were detected by ECL (Amersham). **B**, activation of caspase-2 and -3 and cleavage of PARP in apoptosis induced by TNF α of HeLa cells. 1×10^6 HeLa cells were treated with 10 ng/ml TNF α and 10 μ g/ml cycloheximide for various time periods as indicated. Immunoblotting was performed as described above.

caspase-2 to immunoblot the same lysate samples (Fig. 2, A and B). This anti-C-terminal caspase-2 antibody recognizes the full-length caspase-2 and three additional polypeptides with estimated molecular masses of 44, 14, and 12 kDa, but not the 32–33-kDa doublets, confirming that the 32–33-kDa products do not contain the C-terminal sequence. The 14-kDa product appeared at the same time point of apoptosis as the 32–33-kDa doublets did, suggesting that it is the C-terminal-containing small subunit of caspase-2. The 12-kDa product was detected much later than the 14-kDa, suggesting that the 12-kDa peptide may be a further cleavage product of the 14-kDa product. There was no change for the 44-kDa peptide in apoptosis, suggesting that it is a protein not related to caspase-2 but is cross-recognized by this anti-C-terminal antibody. This observation showed that caspase-2 is activated by several distinct cleavage events, in which cleavage between the large subunit and small subunit occurs first, followed by cleavage(s) between the large subunit and the pro-domain and within the 14-kDa

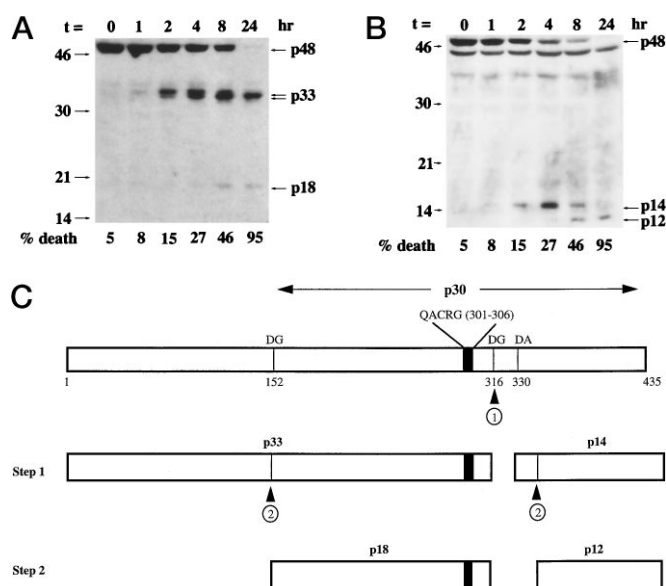


FIG. 2. *A* and *B*, activation of caspase-2 occurs in distinct steps. 5×10^6 Jurkat cells were treated with $1 \mu\text{M}$ staurosporine for various time periods, and the percentages of cell death were determined by trypan blue exclusion assay. The lysates were subjected to immunoblotting analysis using a polyclonal antibody against caspase-2 (*A*), and a polyclonal antibody C-20 specific for caspase-2 C terminus (*B*). *C*, sequential activation of caspase-2. The predicted active site (QACRG) and three potential cleavage sites (Asp residues) are indicated. Fragments of possible structural domains are marked. The fragment between 153 and 316 is the large subunit, which has an approximately molecular mass of 18 kDa, while the fragment between 331 and 435 is the small subunit, which has an approximately molecular mass of 12 kDa. The N-terminal sequence (1–152) is the pro-domain. The p30 is the fragment consisting of the large and small subunits (153–435). Arrows with numbers marked the sequential activation steps of caspase-2. The boxes indicate the cleavage products at different cleavage steps.

C-terminal domain. The possible cleavage sites are summarized in Fig. 2C.

Activation of Caspase-2 Occurs Later than the Activation of Caspase-3-like Proteases when Cells Undergo Apoptosis—To determine possible interactions between caspase-2 and other family members, we compared the temporal activation profiles of caspase-2 and caspase-3. Jurkat cells and HeLa cells were induced to undergo apoptosis by incubation with anti-Fas antibody or staurosporine. The apoptotic cell lysates were immunoblotted with anti-caspase-3 monoclonal antibody as well as antibody specific for poly(ADP-ribose) polymerase (PARP), a substrate of caspase-3. Activation of caspase-3, as indicated by the disappearance of full-length caspase-3 and the appearance of the 89-kDa PARP cleavage product (PARP*), was observed shortly after treatment with apoptotic stimuli at a time point indistinguishable with the first appearance of the caspase-2 32–33-kDa doublets (Fig. 1A). Since the pro-domains of the caspase family often have inhibitory activity (12), and our *in vitro* data suggest that removal of the pro-domain is an essential event for activation of caspase-2 (see below), activation of caspase-2 as marked by the appearance of the 18-kDa cleavage product occurred at a much later time point than that of caspase-3 as marked by the cleavage of PARP (Fig. 1A). Thus, although the first cleavage of caspase-2 occurs at approximately the same time as the activation of caspase-3, activation of caspase-2 did not occur until 2–3 h later.

Caspase-2 Is a Substrate of Caspase-3 in Vitro—When cells undergo apoptosis, caspase-2 may be activated by another caspase(s) and/or by its self-catalytic activity. To address this issue, we tested whether active caspase-2 and other ICE-like proteases are capable of cleaving pro-caspase-2. When we expressed full-length caspase-2 cDNA in *E. coli*, we could not

obtain active protease activity even though 50% of full-length caspase-2 was processed into pro-domain-large subunit and small subunit.² These findings suggest that the N-terminal pro-domain has an inhibitory effect on caspase-2 self-processing, especially on the processing between the pro-domain and large subunit, and that the combination of pro-domain-large subunit and the small subunit is inactive. To obtain active caspase-2 protease, we expressed a fragment (named p30) in *E. coli* containing a deletion of the N-terminal pro-domain 152 amino acid residues. As a control, we created a mutant which contains Cys to Ser mutation in the coding region of the active site pentapeptide QACRG (p30_{C-S}). Using anti-caspase-2 antibodies in Western blot analysis of bacterial lysates, we found that overexpressed wild type p30 was self-processed into 18-kDa and 14-kDa polypeptides, while the p30_{C-S} mutant remained intact, indicating that processing of wild type p30 is due to its own catalytic activity. To determine whether such bacterially expressed caspase-2 p30 was active, we examined its ability to cleave full-length ³⁵S-labeled *in vitro* translated pro-caspase-2. As shown in Fig. 3A, caspase-2 p30 was capable of cleaving full-length caspase-2 into two polypeptides of 34 kDa and 14 kDa, a pattern similar to the *in vivo* results (Fig. 1). To explore the possibility that caspase-2 may be cleaved by another member of the ICE family, we investigated whether active caspase-1 and caspase-3 cleaved pro-caspase-2 *in vitro*. Caspase-1 and caspase-3 cDNA were expressed in *E. coli*, and such caspase-1 and -3-expressing bacterial lysates were found to efficiently cleave pro-IL-1 β and PARP *in vitro*, respectively (data not shown). As shown in Fig. 3A, caspase-3 cleaved ³⁵S-labeled pro-caspase-2 into two polypeptides of 34 kDa and 14 kDa, while caspase-1 cleaved both caspase-2 and caspase-3 very poorly. In contrast, neither caspase-3 nor p30 of caspase-2 cleaved pro-caspase-1. These results suggest that caspase-3 or a caspase-3-like member of the caspase family may act as an activator of caspase-2.

We also determined the abilities of three peptide inhibitors of the caspase family to inhibit ICH-1 protease activity *in vitro*. YVAD-CHO and DEVD-CHO are relatively specific inhibitors of caspase-1-like and caspase-3-like proteases, respectively. DEVD-CHO inhibits caspase-3 with $K_i = 0.52 \text{ nM}$ (37), whereas YVAD-CHO is a very potent inhibitor of caspase-1 ($K_i = 0.76 \text{ nM}$) (2). Addition of YVAD-CMK ($5 \mu\text{M}$), an irreversible inhibitor of caspase-1-like proteases, inhibited the cleavage of pro-caspase-2 by p30 (data not shown). The caspase-2 activity, however, cannot be inhibited by YVAD-CHO (up to $400 \mu\text{M}$, data not shown) and is insensitive to DEVD-CHO; only 50% inhibited at $10 \mu\text{M}$ DEVD-CHO with preincubation (Fig. 3B). In contrast, cleavage of caspase-2 by caspase-3 is much more sensitive to DEVD-CHO than that by caspase-2 itself: 50 nM DEVD-CHO inhibited the cleavage completely (Fig. 3C).

Processing of Caspase-2 and DNA Fragmentation, but Not Loss of Mitochondrial Function, Is Inhibited by Up to $50 \mu\text{M}$ DEVD-CHO—Our *in vitro* cleavage results suggest that caspase-3 or a caspase-3-like protease may act as an activator of caspase-2. To elucidate the mechanism of caspase-2 activation during apoptosis, we examined whether DEVD-CHO inhibited caspase-2 activation and apoptosis *in vivo*. Previous studies have shown that DEVD-CHO can inhibit apoptosis in cultured cells as well as in animals, although the concentrations required are much higher than what is needed to inhibit individual caspases in purified forms (38–42). Jurkat cells were treated with anti-Fas antibody in the presence of different concentrations of DEVD-CHO. Percentages of viable cells were assessed by MTT assay (34), which measures mitochondrial

² H. Li, unpublished data.

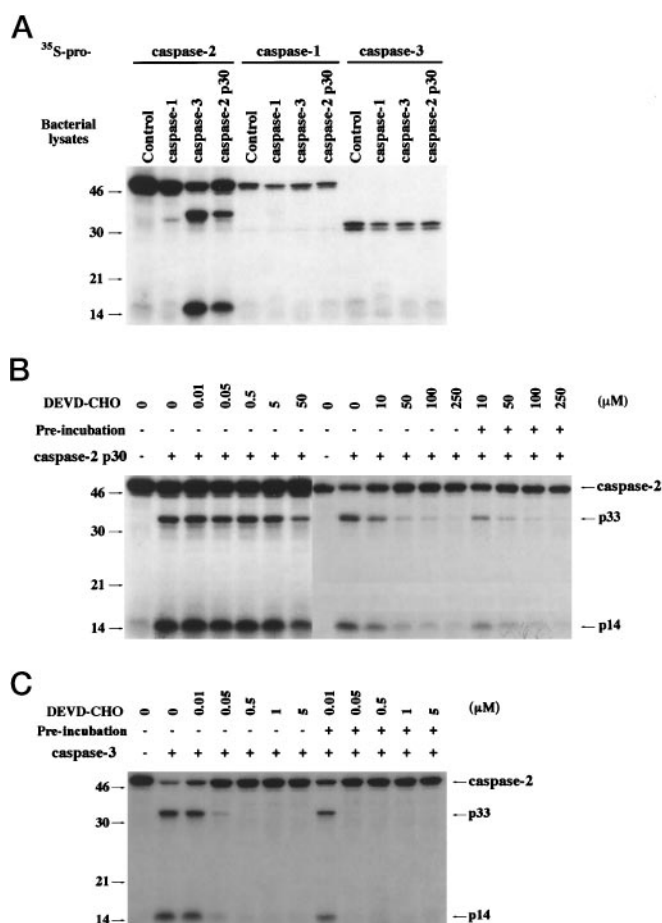


FIG. 3. *In vitro* cleavage of pro-caspase-2 by its active form and caspase-3. A, pro-caspase-2 is cleaved by active caspase-2 and -3, but not by caspase-1. 35 S-labeled pro-caspase-1, -2, and -3 was incubated with 20 μ g of bacterial lysates containing either active caspase-2 p30, caspase-1 or caspase-3 in the reaction buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 10 mM DTT) with protease inhibitors (PMSF and aprotinin) for 1 h at 30 $^{\circ}$ C. The control lysate was caspase-2 p30_{C-S}. B, sensitivity of caspase-2 p30 to inhibition by DEVD-CHO. 35 S-labeled pro-caspase-2 was incubated with the bacterial lysate containing the active caspase-2 p30 for 1 h at 30 $^{\circ}$ C, in the presence or absence of different amounts of DEVD-CHO. Preincubation of DEVD-CHO with caspase-2 p30 was carried out for 15 min at 30 $^{\circ}$ C. C, sensitivity of caspase-3 to inhibition by DEVD-CHO. The experiment was performed as described above, except that the bacterial lysate containing caspase-3 activity was used.

function, and processing of caspase-2 was examined by immunoblotting using anti-caspase-2 polyclonal antibody. As shown in Fig. 4A, approximately 50% of the caspase-2 processing was inhibited by 10 μ M DEVD-CHO, and 90% of caspase-2 processing was inhibited by 50 μ M DEVD-CHO, a concentration that completely inhibited PARP cleavage. In contrast, caspase-3 activation as marked by the disappearance of full-length caspase-3 was not affected by 10 μ M DEVD-CHO, and modestly affected by up to 100 μ M DEVD-CHO (Fig. 4A). These results suggest that although the activity of caspase-3 indicated by PARP cleavage is sensitive to DEVD-CHO, caspase-3 itself is activated by a caspase less sensitive to DEVD-CHO. DNA fragmentation was nearly half inhibited by 10 μ M DEVD-CHO, and almost completely inhibited by 50 μ M DEVD-CHO (Fig. 4B). In contrast, up to 50 μ M DEVD-CHO had no effect on cell viability as measured by MTT assay (Fig. 4). These results showed that loss of mitochondria function, activation of caspases-2 and -3, DNA fragmentation, and cleavage of PARP can be distinguished by their differential sensitivities to the inhibition by DEVD-CHO.

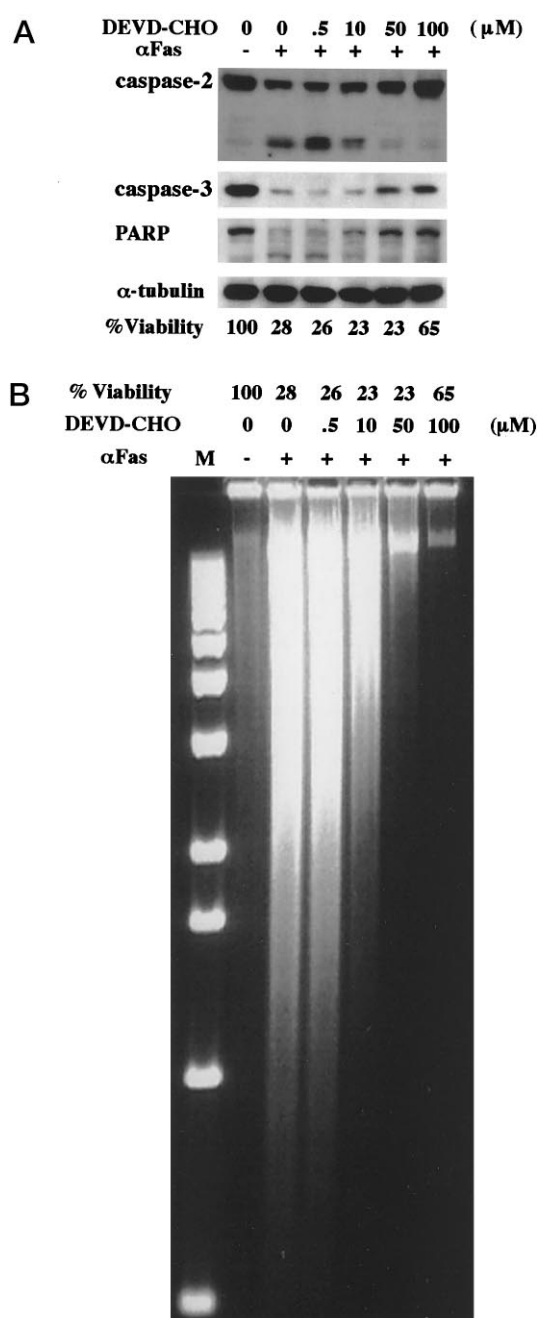


FIG. 4. Inhibition of caspase-2 and -3 activation and DNA fragmentation by DEVD-CHO. A, processing of caspase-2 and -3, and (B) DNA fragmentation assay. 2×10^6 Jurkat cells were treated with 200 ng/ml anti-FAS monoclonal antibody for 20 h, in the presence of DEVD-CHO (0.5, 10, 50, and 100 μ M). The percentage of viability was determined by MTT assay. Processing of caspase-2 was examined by Western blotting using anti-caspase-2 polyclonal antibody. M, 1-kb marker from Life Technologies, Inc.

Activation of ICH-1 in a Cell-free System—To further explore the identity of the upstream activator of caspase-2, we established a cell-free system using staurosporine-induced apoptotic Jurkat cytosolic lysate. Jurkat cells were induced to undergo apoptosis in the presence of 1 μ M staurosporine. Cytosolic extracts at different time points of staurosporine treatment were isolated and incubated with 35 S-labeled *in vitro* translated caspase-2 and PARP for 2 h (Fig. 5A). Cleavage of caspase-2 into 34 and 14 kDa and cleavage of PARP into 89 and 27 kDa in apoptosis induced by staurosporine occurred in a similar time course as to that induced by anti-Fas antibody. Furthermore, cleavage of both PARP and caspase-2 in this cell-free

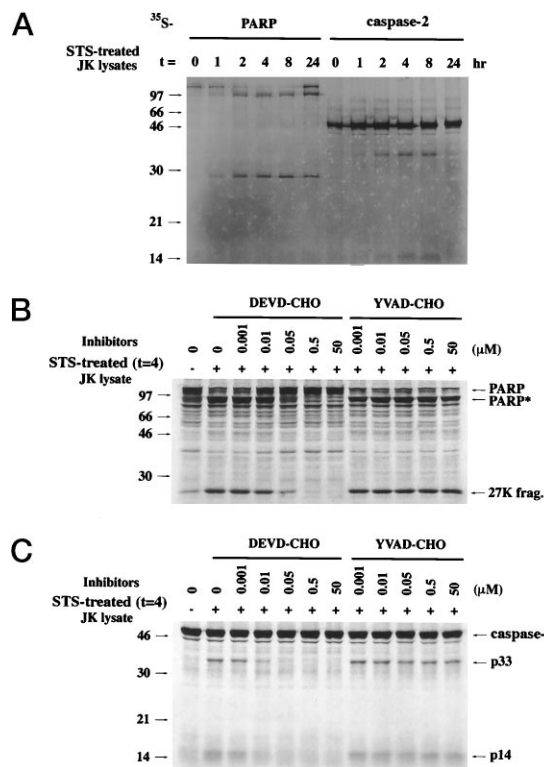


FIG. 5. Caspase-2 is cleaved in a cell-free system. A, caspase-2 is cleaved by staurosporine (STS)-treated Jurkat cytosolic lysates. 1×10^8 Jurkat cells were treated with $1 \mu\text{M}$ staurosporine for different time periods indicated, and cytosolic lysates were prepared as described under "Materials and Methods." ^{35}S -Labeled-pro-caspase-2 and PARP were incubated with $30 \mu\text{g}$ of lysates for 2 h at 30°C . B and C, processing of PARP (B), and processing of caspase-2 (C), by staurosporine-treated lysates, is sensitive to DEVD-CHO, not YVAD-CHO. ^{35}S -Labeled pro-caspase-2 and PARP were incubated with staurosporine-treated Jurkat lysates (4 h of treatment) in the presence and absence of DEVD-CHO or YVAD-CHO (0.001, 0.01, 0.05, 0.5, or $50 \mu\text{M}$, respectively).

system was sensitive to DEVD-CHO (50 nM) but insensitive to YVAD-CHO ($50 \mu\text{M}$) (Fig. 5, B and C). These results again suggest that caspase-2 is activated by caspase-3 or caspase-3-like proteases during apoptosis.

Determination of Cleavage Sites of Caspase-2 Processing—Proteolytic activation of caspase proteases involves cleavage of specific Asp residues in the precursor peptides. Based upon the homology between caspase-2 and 1 and the consensus sequence of caspase-3 cleavage, several Asp residues in caspase-2 are candidates for processing sites (Asp-83, Asp-99, Asp-118, Asp-120, Asp-152, Asp-316, and Asp-330; only DNKD¹⁵²G¹⁵³ and DQQD³¹⁶G³¹⁷ has caspase-3 cleavage consensus sequence). To determine the processing sites of caspase-2 *in vitro*, we mutated Asp residues at positions 316 and 330 to Glu (D316E and D330E), and *in vitro* cleavage assays were performed using these two mutants. As shown in Fig. 6, the mutation at Asp316 (D316E) completely blocked the pro-caspase-2 cleavage event by either caspase-2 and caspase-3 expressing bacterial lysate, or staurosporine-treated Jurkat apoptotic cytosolic lysate (data not shown), whereas the D330E mutation appears to alter the cleavage site, suggesting that Asp-316 was the primary cleavage site of caspase-2 by its activator. The caspase-2 p30 double mutant bearing D316E/D330E was incapable of self-processing and cleaving pro-caspase-2, indicating that processing at Asp-316 is essential for caspase-2 proteolytic activity (data not shown).

Caspase-2 Is Activated in CTL-mediated Apoptosis—CTL-mediated cytotoxicity, the major cellular defense against virus-

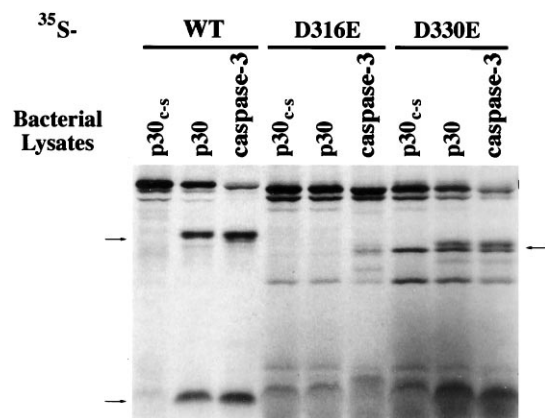


FIG. 6. D316 is the primary cleavage site for caspase-2 processing. ^{35}S -Labeled pro-caspase-2 and its two mutants (D316E and D330E) were incubated with bacterial lysates containing caspase-2 p30_{C-S}, caspase-2 p30, or caspase-2 for 1 h at 30°C . Processing of caspase-2 was examined by SDS-PAGE and autoradiography. The arrows on the left indicate the correct cleavage products of wild type caspase-2, whereas the arrow on the right indicates one of the cleavage products of D330E mutant by caspase-2 p30 and caspase-2, with altered specificity.

infected and tumorigenic cells, is executed through two mechanisms: perforin-granzyme B pathway (Ca^{2+} -dependent) and Fas signaling pathway (Ca^{2+} -independent) (43, 44). Previous studies have shown that CrmA, a specific inhibitor of caspase-1, can inhibit CTL-mediated apoptosis, primarily by blocking the Fas pathway (29). Granzyme B can cleave and directly activate caspase-3 (45). It is of particular interest to examine whether caspase-2 is activated in CTL-mediated apoptosis and is activated by granzyme B directly. CTL-resistant (P815) and CTL-sensitive target cells were incubated with CTL clone F3B4 in a ratio of 1:1. Caspase-2 is barely expressed in CTL, but highly expressed in target cells (Fig. 7A). Caspase-2 was fully processed within 45 min in positive target cells EL4, whereas it remained intact in negative control cells P815, indicating that caspase-2 may also play a role in CTL-mediated apoptosis. We could not observe processing products of caspase-2 since our anti-caspase-2 polyclonal antibody was generated against human caspase-2 and does not recognize processed mouse caspase-2. To determine if granzyme B can directly activate caspase-2, we determined if purified granzyme B (54) may cleave *in vitro* translated ^{35}S -labeled caspase-2. Such analysis showed that although granzyme B cleaves caspase-3 efficiently, it cannot cleave caspase-2 (Fig. 7B). Thus, activation of caspase-2 by CTL is most likely to be mediated through the Fas pathway or indirectly by another caspase(s) activated by granzyme B rather than granzyme B itself.

DISCUSSION

We have demonstrated that caspase-2 (NEDD-2/ICH-1), a member of the ICE family, is activated when cells are induced to undergo apoptosis by diverse stimuli such as anti-Fas antibody, $\text{TNF}\alpha$, and staurosporine. When cells are induced to undergo apoptosis, endogenous caspase-2 is first cleaved into three fragments of 32–33 and 14 kDa, which are then processed further into 18-kDa and 12-kDa active subunit. When overexpressed in bacteria, the fragment of caspase-2 without its N-terminal pro-domain was cleaved into two peptides of 18 and 12 kDa, which are enzymatically active, similar to what has been reported (46). The 18-kDa polypeptide detected by anti-caspase-2 antibody in apoptotic cells is likely to be the large subunit of active caspase-2. Taken together, our *in vitro* and *in vivo* observations strongly suggest that caspase-2 is indeed activated when cells undergo apoptosis.

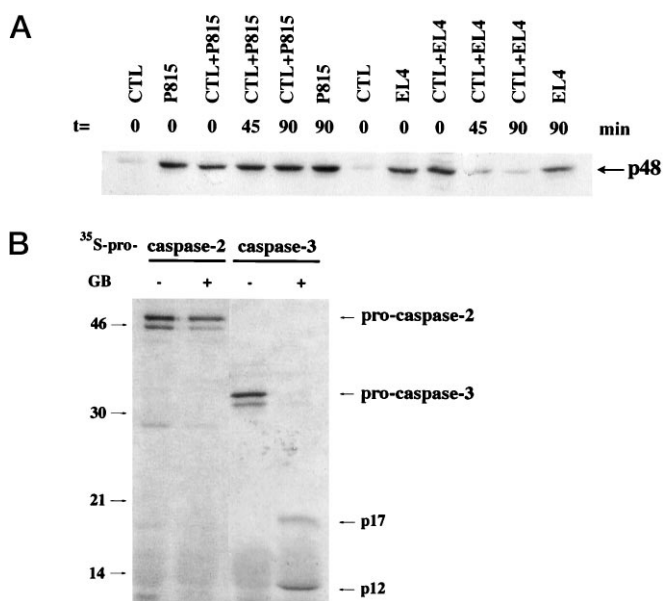


FIG. 7. Activation of caspase-2 in apoptosis induced by CTL. *A*, caspase-2 is processed in CTL-mediated apoptosis. CTL and target cells were incubated at 1:1 ratio for 45 or 90 min. The total cell lysates were subjected to SDS-PAGE and immunoblotting using caspase-2 polyclonal antibody. P815 is resistant, and EL4 is sensitive to CTL-mediated apoptosis. *B*, granzyme B can cleave caspase-3, but not caspase-2 directly *in vitro*. ³⁵S-Labeled pro-caspase-2 and -3 were incubated with 20 ng of purified GB for 1 h at 30 °C in a reaction buffer containing protease inhibitors (PMSF and aprotinin). Arrows indicate the full-length caspase-2 and -3, and their processing products.

The mechanism of activation of ICE/CED-3 cysteine proteases remains unclear so far. Two possible mechanisms, which are not mutually exclusive, may be involved. The first mechanism is that each member of the caspase family is activated through self-catalytic cleavage upon dissociation with a putative inhibitor(s). The evidence supporting this notion is that several members, when overexpressed *in vitro*, are capable of undergoing self-cleavage to generate active enzymes (10, 12, 47). The second possible mechanism is cross-activation whereby one caspase activates another one(s). We found that while caspase-2 activity *in vitro* is much less sensitive to the inhibition by DEVD-CHO than that of caspase-3, the activation of caspase-2 in cells, as indicated by the cleavage of pro-caspase-2, is as sensitive to the inhibition by DEVD-CHO as that of cleavage of PARP, an indicator of caspase-3-like activity. Our results suggest that caspase-2 is most likely to be activated by a caspase-3-like activity rather than by a self-activation mechanism. It has been shown that in *in vitro* assay systems, caspase-4 (TX/ICH-2) can process both pro-caspase-4 and pro-caspase-1 (10), and caspase-1 can process and activate pro-caspase-1 and caspase-3 (8). It is not clear, however, whether such cross-activation indeed occurs in cells undergoing apoptosis. Our study demonstrated that *in vivo* one member of the caspase family, caspase-2, is activated by another member of the caspase family, a caspase-3-like protease(s), when cells are induced to undergo apoptosis by staurosporine and anti-Fas antibody. Dr. Shige Nagata's laboratory has shown that when cells are induced to undergo apoptosis by anti-Fas antibody, there is a sequential activation of caspase-1-like and caspase-3-like proteases (48). Our results extended their observation by revealing downstream targets of the caspase-3-like proteases. The observation that caspase-2 was processed in CTL-mediated apoptosis, but granzyme B cannot cleave caspase-2 directly, also suggests that other factors mediate caspase-2 activation in perforin-granzyme B killing. Taken together, we propose a model of sequential activation involving

three subfamilies of ICE/CED-3 proteases in the execution of programmed cell death. In this model, when cells are stimulated with a death signal such as anti-Fas antibody, a caspase-1-like protease(s) is activated first, followed by activation of a caspase-3-like protease(s) that may be mediated by the caspase-1-like activity, and then a caspase-3-like protease(s) activates caspase-2. We do not know, however, the exact identities of the upstream caspase-1- and caspase-3-like activity. Further studies using mutant mice that are defective in one or more members of the caspase family proteases are needed to clarify these questions.

Caspase-3 protease is activated by cleavage events at Asp-28/Ser-29 (between N-terminal pro-domain) and Asp-175/Ser-176 (between the large and the small subunits) to generate a large subunit of 17 kDa and a small subunit of 12 kDa (7), whereas pro-caspase-1 is activated through four cleavage events: two cleavages between the N-terminal prodomain (Asp-103/Ser-104 and Asp-119/Asn-120) and two between the large and small subunits (Asp-297/Ser-298 and Asp-316/Ala-317) (2). The temporal sequences of proteolytic cleavages during caspase-1 and -3 activation are not clear. We showed here that activation of caspase-2 occurs in distinct cleavage steps. The timing of the first cleavage between the large subunit and the small subunit coincides with the activation of caspase-3 and cleavage of PARP. This cleavage is inhibitable by DEVD-CHO *in vivo* and *in vitro*, although the active caspase-2 itself is much less sensitive to this inhibitor than that of caspase-3. These two observations suggest strongly that this first cleavage of caspase-2 is carried out by caspase-3 or a caspase-3-like protease. Our *in vitro* data indicate that a single cleavage between the large subunit and the small subunit of caspase-3, however, is insufficient to activate caspase-2. The second cleavage of caspase-2, between the pro-domain and the large subunit, occurs much later at 4 h, when 25% of cells are dead as estimated by MTT assay. Neither caspase-3 nor active caspase-2 can carry out this second cleavage *in vitro*, suggesting that this cleavage is executed by an uncharacterized protease.

Apoptosis is usually measured by MTT assay, DNA fragmentation, or trypan blue exclusion (49). Each of these procedures measures a different parameter of cell viability. Trypan blue exclusion measures the integrity of cell membrane or permeability change. Disruption of the cytoplasmic membrane occurs relatively late in apoptosis. DNA fragmentation, representing an alteration in nuclei, occurs much earlier than changes in cell membrane permeability (our unpublished observation). The MTT assay is a quantitative colorimetric assay based on reduction of a tetrazolium salt, MTT. MTT is reduced within the active mitochondria of living cells by the enzyme succinate dehydrogenase (50). The salt is reduced to an insoluble blue formazan product in living cells but not in the mitochondria or cellular debris of dead cells. 70–80% of mitochondrial MTT reduction occurs subsequent to transfer of electrons from cytochrome *c* to cytochrome oxidase, but prior to the point of azide inhibition (51). Loss of mitochondrial function, a process beginning with a decrease in mitochondrial transmembrane potential, followed by mitochondrial uncoupling and generation of reactive oxygen species, precedes nuclear alteration (52). Recently, release of cytochrome *c* from mitochondria has been shown to be an early and essential step of apoptosis in a cell-free system induced by dATP (53). Our data showed here that there is a concentration of DEVD-CHO (50 μM), which inhibits the cleavage and activation of caspase-2 by a caspase-3-like activity and DNA fragmentation but does not alter viability as measured by MTT, suggesting that DEVD-CHO at that dose can block activation of the caspase family members such as caspase-2 but cannot block loss of mitochondrial func-

tion in apoptosis induced by anti-Fas antibody. These results indicate that activation of caspase-2 by a caspase-3-like activity is separable from the loss of mitochondrial function. Higher doses of DEVD-CHO, however, can inhibit loss of mitochondrial function as measured by MTT. Since the subfamily of caspase-1-like proteases that are mostly closely related to caspase-1 requires higher concentrations of DEVD-CHO for inhibition, this result suggests that there is an caspase-1-like activity further upstream from loss of mitochondrial function. This result is consistent with the report by Enari *et al.* (48), who showed that activation of an caspase-1-like activity precedes the activation of caspase-3-like activities in apoptosis induced by anti-Fas activity. It is not clear, however, in lieu of the recent report of caspase-8 (FLICE/MACH), an caspase-3-like protease containing MORT domain that allows direct coupling to the Fas receptor upon activation, the exact identity of this caspase-1-like activity.

REFERENCES

- Cerretti, D. P., Kozlosky, C. J., Mosley, B., Nelson, N., Van Ness, K., Greenstreet, T. A., March, C. J., Kronheim, S. R., Druck, T., Cannizzaro, L. A., Huebner, K., and Black, R. A. (1992) *Science* **256**, 97–100
- Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., Aunins, J., Elliston, K. O., Ayala, J. M., Casano, F. J., Chin, J., Ding, G. J.-F., Egger, L. A., Gaffney, E. P., Limjoco, G., Palyha, O. C., Raju, S. M., Rolando, A. M., Salley, J. P., Yamin, T.-T., Lee, T. D., Shively, J. E., MacCross, M., Mumford, R. A., Schmidt, J. A., and Tocci, M. J. (1992) *Nature* **356**, 768–774
- Miura, M., Zhu, H., Rotello, R., Hartwig, E. A., and Yuan, J. (1993) *Cell* **75**, 653–660
- Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993) *Cell* **75**, 641–652
- Kumar, S., Kinoshita, M., Noda, M., Copeland, N. G., and Jenkins, N. A. (1994) *Genes & Dev.* **8**, 1613–1626
- Wang, L., Miura, M., Bergeron, L., Zhu, H., and Yuan, J. (1994) *Cell* **78**, 739–750
- Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1994) *J. Biol. Chem.* **269**, 30761–30764
- Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salveson, G. S., and Dixit, V. M. (1995) *Cell* **81**, 801–809
- Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1995) *Cancer Res.* **55**, 2737–2742
- Faucheu, C., Diu, A., Chan, A. W., Blanchet, A. M., Miossec, C., Herve, F., Collard-Dutilleul, V., Gu, Y., Aldape, R. A., Lippke, J. A., Rocher, C., Su, M. S.-S., Livingston, D. J., Hercend, T., and Lalanne, J. (1995) *EMBO J.* **14**, 1914–1922
- Kamens, J., Paskind, M., Hugunin, M., Talanian, R. V., Allen, H., Banach, D., Bump, N., Hackett, M., Johnston, C. G., Li, P., Mankovich, J. A., Terranova, M., and Ghayur, T. (1995) *J. Biol. Chem.* **270**, 15250–15256
- Munday, N. A., Vaillancourt, J. P., Ali, A., Casano, F. J., Miller, D. K., Molineaux, S. M., Yamin, T.-T., Yu, V. L., and Nicholson, D. W. (1995) *J. Biol. Chem.* **270**, 15870–15876
- Duan, H., Chinnaiyan, A. M., Hudson, P. L., Wing, J. P., He, W.-W., and Dixit, V. M. (1996) *J. Biol. Chem.* **271**, 1621–1625
- Fernandes-Alnemri, T., Takahashi, A., Armstrong, R., Krebs, J., Fritz, L., Tomaselli, K. J., Wang, L., Yu, Z., Croce, C. M., Salveson, G., Earnshaw, W. C., Litwack, G., and Alnemri, E. S. (1995) *Cancer Res.* **55**, 6045–6052
- Lippke, J. A., Gu, Y., Sarnecki, C., Caron, P. R., and Su, M. S.-S. (1996) *J. Biol. Chem.* **271**, 1825–1828
- Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996) *Cell* **85**, 803–815
- Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Kreammer, P. H., Peter, M. E., and Dixit, V. M. (1996) *Cell* **85**, 817–827
- Fernandes-Alnemri, T., Armstrong, R. C., Krebs, J., Srinivasula, S. M., Wang, L., Bullrich, F., Fritz, L. C., Trapani, J. A., Tomaselli, K. J., Litwack, G., and Alnemri, E. S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 7464–7469
- Duan, H., Orth, K., Chinnaiyan, A. M., Poirier, G. G., Froelich, C. J., He, W.-W., and Dixit, V. M. (1996) *J. Biol. Chem.* **271**, 16720–16724
- Srinivasula, S. M., Fernandes-Alnemri, T., Zangrilli, J., Robertson, N., Armstrong, R. C., Wang, L., Trapani, J. A., Tomaselli, K. J., Litwack, G., Alnemri, E. S. (1996) *J. Biol. Chem.* **271**, 27099–27106
- Kumar, S. (1995) *Trends Biochem. Sci.* **20**, 198–202
- Martin, S. J., and Green, D. R. (1995) *Cell* **82**, 349–352
- Steller, H. (1995) *Science* **267**, 1445–1449
- Gagliardini, V., Fernandez, P.-A., Lee, R. K. K., Drexler, H. C. A., Rotello, R. J., Fishman, M. C., and Yuan, J. (1994) *Science* **263**, 826–828
- Enari, M., Hug, H., and Nagata, S. (1995) *Nature* **375**, 78–81
- Los, M., Van de Craen, M., Penning, L. C., Schenk, H., Westendorp, M., Baeuerle, P. A., Dröge, W., Krammer, P. H., Fiers, W., and Schulze-Osthoff, K. (1995) *Nature* **375**, 81–83
- Miura, M., Friedlander, R. M., and Yuan, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 8318–8322
- Tewari, M., and Dixit, V. M. (1995) *J. Biol. Chem.* **270**, 3255–3260
- Tewari, M., Telford, W. G., Miller, R. A., and Dixit, V. M. (1995) *J. Biol. Chem.* **270**, 22705–22708
- Kuida, K., Lippke, J. A., Ku, G., Harding, M. W., Livingston, D. J., Su, M. S., and Flavell, R. A. (1995) *Science* **267**, 2000–2003
- Milligan, C. E., Prevette, D., Yaginuma, H., Homma, S., Cardwell, C., Fritz, L. C., Tomaselli, K. J., Oppenheim, R. W., and Schwartz, L. M. (1995) *Neuron* **15**, 385–393
- Kumar, S., Tomooka, Y., and Noda, M. (1992) *Biochem. Biophys. Res. Commun.* **185**, 1155–1161
- Mosmann, T. (1983) *J. Immunol. Methods* **65**, 55–63
- Eastman, A. (1995) *Methods Cell Biol.* **46**, 41–55
- Chow, S. C., Weis, M., Kass, G. E., Holmstrom, T. H., Eriksson, J. E., and Orrenius, S. (1995) *FEBS Lett.* **364**, 134–138
- Jacobson, M., Burne, J., and Raff, M. (1994) *EMBO J.* **13**, 1899–1910
- Margolin, N., Raybuck, S. A., Wilson, K. P., Chen, W., Fox, T., Gu, Y., and Livingston, D. J. (1997) *J. Biol. Chem.* **272**, 7223–7228
- Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T., Yu, V. L., and Miller, D. K. (1995) *Nature* **376**, 37–43
- Shi, L., Chen, G., MacDonald, G., Bergeron, L., Li, H., Miura, M., Rotello, R. J., Miller, D. K., Li, P., Seshadri, T., Yuan, J., and Greenberg, A. H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 11002–11007
- Anel, A., Gamen, S., Alava, M. A., Schmitt-Verhulst, A. M., Pineiro, A., and Naval, J. (1997) *J. Immunol.* **158**, 1999–2006
- Hasegawa, J., Kamada, S., Kamiike, W., Shimizu, S., Imazu, T., Matsuda, H., and Tsujimoto, Y. (1996) *Cancer Res.* **56**, 1713–1718
- Hara, H., Friedlander, R. M., Gagliardini, V., Ayata, C., Fink, K., Huang, Z., Shimizu-Sasamata, M., Yuan, J., and Moskowitz, M. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2007–2012
- Kagi, D., Vignaux, F., Ledermann, B., Burki, K., Pepsraetere, V., Nagata, S., Hengartner, H., and Golstein, P. (1994) *Science* **265**, 528–530
- Lowin, B., Ahne, M. C., and Tschopp, J. (1994) *Nature* **370**, 650–652
- Darmon, A. J., Nicholson, D. W., and Bleackley, R. C. (1995) *Nature* **377**, 446–448
- Xue, D., Shaham, S., and Horvitz, H. R. (1996) *Genes & Dev.* **10**, 1073–1083
- Ramage, P., Cheneval, D., Chvei, M., Graff, P., Hemmig, R., Heng, R., Kocher, H. P., Mackenzie, A., Memmert, K., Revesz, L., and Wishart, W. (1995) *J. Biol. Chem.* **270**, 9378–9383
- Enari, M., Talanian, R. V., Wong, W. W., and Nagata, S. (1996) *Nature* **380**, 723–726
- McGahan, A., Martin, S., Bissonnette, R., Mahboubi, A., Shi, Y., Mogil, R., Nishioka, W., and Green, D. R. (1995) *Methods Cell Biol.* **46**, 153–181
- Carmichael, J., DeGraff, W., Gazdcar, A., Minna, J., and Mitchell, J. (1987) *Cancer Res.* **47**, 943–946
- Berridge, M., and Tan, A. (1993) *Arch. Biochem. Biophys.* **303**, 474–482
- Kroemer, G., Petit, P., Zamzami, N., Vayssiere, J., and Mignotte, B. (1995) *FASEB J.* **9**, 1277–1287
- Liu, X., Kim, C., Yang, J., Jemmerson, R., and Wang, X. (1996) *Cell* **86**, 147–157
- Shi, L., Kam, C. M., Powers, J. C., Aebersold, R., and Greenberg, A. H. (1992) *J. Exp. Med.* **176**, 1521–1529

Activation of Caspase-2 in Apoptosis

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