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Activation of Caspase-2 in Apoptosis*

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Members of the CED-3 interleukin-1β-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 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 μM 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 μM) inhibit both. In comparison, although the activity of caspase-3 is very sensitive to the inhibition of DEVD-CHO (<50 μM), inhibition of caspase-3 activation as marked by processing of the proform requires more than 100 μM 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-1β-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/Apoptain) (7, 8, 39), caspase-6 (Mch-2) (9), caspase-4 (TX/Ich-2/ICEeII) (10–12), caspase-5 (ICEeIII) (13), caspase-7 (Mch-3/CMH-1/ICE-LAP3) (13–15), caspase-8 (FLICE/MACH/Mch-5) (16–18), caspase-10 (Mch-4) (19), 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-1L and ICH-1S, which have antagonistic effects on cell death. ICH-1L (435 amino acids) contains sequence homologous to both p20 and p10 subunits of ICE, while ICH-1S (312 amino acids) is a truncated version of ICH-1L, containing only the p20 region. Previous studies of Ich-1 in our laboratory revealed that overexpression of Ich-1L induces programmed cell death, while overexpression of Ich-1S 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 stage development 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-1L and Ich-1S can be detected in heart, kidney, and embryonic and adult

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†† The abbreviations used are: ICE, interleukin-1β-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, being highest in embryonic brain, and only Ich-1, 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 demonstrated 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-(dimethylthiazol-2-yl)-2,5-diphenyl-yl]tetrazolium 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. The cell lines were transfected with plasmids expressing caspase-2 or caspase-1, or transfected with a control plasmid. Protein translations of 35S-labeled proteins were obtained by centrifugation at 12,000 g for 15 min at 4 °C, 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 [35S]labeled proteins were done by using the TNT-coupled transcription/translation kit (Promega). The reactions 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, 1% Nonidet P-40, and 20 μg/ml PMSF), and sonicated. The supernatant after centrifugation at 14,000 × 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 TNFα (BD & Systems, Minneapolis, MN) and cycloheximide. The protein concentration 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 × 10⁶ cells (50 μl) 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 μl 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 viability was determined colorimetrically by measuring absorbance of resorufin (Molecular Devices, Sunnyvale, CA) and SoftMax software to measure absorbance to 570–560 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 × 50 cm) gel tank. After the gel solidified, the top section of gel immediately above the comb was removed, and filled with 1% agarose, 2% SDS, 64 μg/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 μl of sample buffer (5% glycerol, 5 mM Tris, pH 8.0, 0.05% bromphenol blue, and 5 μg/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 μg/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 with 10% polyacrylamide gel and then transferred to a nylon membrane (Magna, 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; SEYCTLCHR-LYLFQHPFT) with a dilution of 1:2000, 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 × 10⁸) were treated with 1 μM staurosporine for various time periods, and cytosolic lysates were prepared as described with minor modifications (35). Briefly, cells were washed twice with cold RPMI 1640, and resuspended in 400 μl 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 μg/ml leupeptin, 0.5 μg/ml aprotinin). After four cycles of freezing and thawing, crude extracts were obtained by centrifugation at 12,000 × g for 15 min at 4 °C. The crude extracts were further centrifuged at 100,000 × 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.

**Activation of Caspase-2 in Apoptosis**

21011
that were induced to die by TNF.

Processing of caspase-2 was also detected in HeLa cells. 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. Over night 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, to induce 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 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 TNFα 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.

A Jurkat Cell

| caspase-2 | PARP | α-tubulin |
|----------|------|-----------|
| time (hrs) | % death | % death | % death |
| 0 | 5 | 5 | 5 |
| 1 | 10 | 10 | 10 |
| 2 | 25 | 25 | 25 |
| 4 | 30 | 30 | 30 |
| 8 | 65 | 65 | 65 |
| 24 | 95 | 95 | 95 |

B HeLa Cell

| caspase-2 | PARP | α-tubulin |
|----------|------|-----------|
| time (hrs) | % death | % death | % death |
| 0 | 5 | 5 | 5 |
| 4 | 10 | 10 | 10 |
| 8 | 25 | 25 | 25 |
| 24 | 70 | 70 | 70 |

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 × 10⁶ Jurkat cells were treated with either 10 ng/ml anti-Fas antibody (CT-11) or 0.1 μM staurosporine (STS) in the presence of 50 ng/ml cycloheximide (CHX) for various time periods as indicated. Aliquots of the total cell lysates were subjected to 15% 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 TNFα of HeLa cells. 1 × 10⁶ HeLa cells were treated with 10 ng/ml TNFα and 10 μg/ml cycloheximide for various time periods as indicated. Immunoblotting was performed as described above.
Activation of Caspase-2 in Apoptosis

When cells undergo apoptosis, caspase-2 may be activated by another member of the ICE family. We investigated whether active caspase-1 and caspase-3 cleaved 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. 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-1β 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 $K_i = 0.52$ nM (37), whereas YVAD-CHO is a very potent inhibitor of caspase-1 ($K_i = 0.76$ nM) (2). Addition of YVAD-CMK (5 μM), 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 μM, data not shown) and is insensitive to DEVD-CHO; only 50% inhibited at 10 μM 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 μM 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 vitro. 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

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2 H. Li, unpublished data.
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 μM DEVD-CHO, and 90% of caspase-2 processing was inhibited by 50 μM 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 μM DEVD-CHO, and modestly affected by up to 100 μM 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 μM DEVD-CHO, and almost completely inhibited by 50 μM DEVD-CHO (Fig. 4B). In contrast, up to 50 μM DEVD-CHO had no effect on cell viability as measured by MTT assay (Fig. 4A). 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.

**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 μM staurosporine. Cytosolic extracts at different time points of staurosporine treatment were isolated and incubated with 35S-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
system was sensitive to DEVD-CHO (50 nM) but insensitive to
YVAD-CHO (50 μM) (Fig. 5, B and C). These results again
suggest that caspase-2 is activated by caspase-3 or caspase-3-
like proteases during apoptosis.

**FIG. 5. Caspase-2 is cleaved in a cell-free system.** A, caspase-2 is
cleaved by staurosporine (STS)-treated Jurkat cytosolic lysates. 1 × 10⁶
Jurkat cells were treated with 1 μM staurosporine for different time
periods indicated, and cytosolic lysates were prepared as described
under "Materials and Methods." 35S-Labeled-pro-caspase-2 and PARP
were incubated with 30 μg of lysates for 2 h at 30°C. B and C, pro-
cessing of PARP (B), and processing of caspase-2 (C), by staurosporine-
treated lysates, is sensitive to DEVD-CHO, not YVAD-CHO. 35S-
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 μM,
respectively).

**FIG. 6. D316 is the primary cleavage site for caspase-2 processing.**
35S-Labeled pro-caspase-2 and its two mutants (D316E and
D330E) were incubated with bacterial lysates containing caspase-2
p30 (32), caspase-2 p30, or caspase-2 for 1 h at 30°C. Processing of
caspase-2 was examined by SDS-PAGE and autoradiography. The ar-
rows 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.

**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 anti-
body, TNFα, and staurosporine. When cells are induced to
undergo apoptosis, endogenous caspase-2 is first cleaved into
two fragments of 32–33 and 14 kDa, which are then processed
further into 18-kDa and 12-kDa active subunits. When overex-
pressed in bacteria, the fragment of caspase-2 without its N-
terminus 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.
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-
tation 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.

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