

Role of Ced-3/ICE-Family Proteases in Staurosporine-induced Programmed Cell Death

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Abstract. In the accompanying paper by Weil et al. (1996) we show that staurosporine (STS), in the presence of cycloheximide (CHX) to inhibit protein synthesis, induces apoptotic cell death in a large variety of nucleated mammalian cell types, suggesting that all nucleated mammalian cells constitutively express all of the proteins required to undergo programmed cell death (PCD). The reliability of that conclusion depends on the evidence that STS-induced, and (STS+CHX)-induced, cell deaths are bona fide examples of PCD. There is rapidly accumulating evidence that some members of the Ced-3/Interleukin-1 β converting enzyme (ICE) family of cysteine proteases are part of the basic machinery of PCD. Here we show that Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), a cell-permeable, irreversible, tripeptide inhibitor of some of

these proteases, suppresses STS-induced and (STS+CHX)-induced cell death in a wide variety of mammalian cell types, including anucleate cytoplasts, providing strong evidence that these are all bona fide examples of PCD. We show that the Ced-3/ICE family member CPP32 becomes activated in STS-induced PCD, and that Bcl-2 inhibits this activation. Most important, we show that, in some cells at least, one or more CPP32-family members, but not ICE itself, is required for STS-induced PCD. Finally, we show that zVAD-fmk suppresses PCD in the interdigital webs in developing mouse paws and blocks the removal of web tissue during digit development, suggesting that this inhibition will be a useful tool for investigating the roles of PCD in various developmental processes.

PROGRAMMED cell death (PCD)¹ is the process whereby cells activate an intrinsic death program and kill themselves. Two lines of evidence suggest that the death program is similar in all animal cells. First, the morphological features of PCD, termed apoptosis, are generally similar in all animals that have been studied: in most cases the nucleus condenses, the cell shrinks and often fragments, and the cells or fragments are rapidly phagocytosed by neighboring cells or by macrophages (Wyllie et al., 1980). Second, two genes involved in PCD in the nematode *C. elegans* have functional and structural homologues in mammalian cells. *Ced-3*, which is required for PCD in the nematode (Ellis and Horvitz, 1986), encodes a cysteine protease that is homologous to the IL-1 β converting enzyme (ICE) family of cysteine proteases in mammals (Yuan et al., 1993), some of which are involved in PCD in mammalian

cells: overexpression of either Ced-3 or ICE can induce PCD in mammalian cells (Gagliardini et al., 1994; Miura et al., 1993), as can overexpression of other Ced-3/ICE family members (Faucheu et al., 1995; Fernandes-Alnemri et al., 1994, 1995a; Kamens et al., 1995; Kumar et al., 1994; Munday et al., 1995; Wang et al., 1994), while protein and peptide inhibitors of these proteases can suppress PCD in many circumstances (for reviews see Martin and Green, 1995; Thornberry et al., 1995). *Ced-9*, which inhibits PCD in the nematode, encodes a protein that is homologous to the Bcl-2 protein, which inhibits PCD in mammalian cells (Hengartner and Horvitz, 1994); the human *bcl-2* gene can even inhibit PCD in the nematode. These findings suggest that the death program and at least part of its regulation have been conserved in evolution from nematode worms to mammals.

In the present study we have examined the role of the Ced-3/ICE family of proteases in PCD induced by high concentrations of the broad-spectrum protein kinase inhibitor staurosporine (STS). In other studies we have used STS-induced PCD to investigate some fundamental features of PCD in mammalian cells. STS has several advantages as a PCD inducer. In the presence of cycloheximide (CHX) to inhibit protein synthesis, it induces PCD in all of the nucleated mammalian cell types that we and others

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1. *Abbreviations used in this paper:* CHX, cycloheximide; ICE, interleukin-1 β converting enzyme; PARP, poly-(ADP ribose) polymerase; PCD, programmed cell death; PI, propidium iodide; STS, staurosporine.

have tested, including normal and transformed cells in cell culture (Bertrand et al., 1994; Ishizaki et al., 1993, 1994, 1995; Jacobson et al., 1993, 1994), and developing and adult cells in explant culture (accompanying paper by Weil et al. [1996]). As inhibitors of protein synthesis potentiate STS-induced PCD, it is clear that PCD does not require the synthesis of new proteins, although the activation of the death program by some other inducers of PCD clearly does so (Duke and Cohen, 1986; Martin et al., 1988; Oppenheim et al., 1990; Tata, 1966). We concluded from these studies that all of the proteins required to execute the death program are constitutively present in all nucleated mammalian cells (accompanying paper by Weil et al. [1996]).

We have also used STS-induced PCD to show that both PCD and Bcl-2 protection can occur in the absence of a nucleus (Jacobson et al., 1994), mitochondrial respiration (Jacobson et al., 1993), or reactive oxygen species (Jacobson and Raff, 1995). These findings led us to suggest that PCD is orchestrated by a cytoplasmic regulator that acts in parallel on multiple intracellular targets to coordinate the many intracellular events that constitute PCD (Jacobson et al., 1994; Jacobson and Evan, 1994). The strongest candidate for such a cytoplasmic regulator is one or more members of the Ced-3/ICE family of proteases.

Two characteristic features of this family of proteases are that they cleave their target proteins after specific aspartic acids (Nicholson et al., 1995; Tewari et al., 1995; Thornberry et al., 1992; Xue and Horvitz, 1995), and they are themselves activated by cleavage after specific aspartic acids, forming two subunits that together form the active site of the enzyme (Faucheu et al., 1995; Tewari et al., 1995; Thornberry et al., 1992). In vitro at least, some of these proteases can activate themselves (Faucheu et al., 1995; Thornberry et al., 1992), and some can activate other family members (Faucheu et al., 1995; Tewari et al., 1995), suggesting that they can act in a cascade. ICE itself seems not to be required for most PCDs in vivo, as mice with targeted disruptions of the *ice* gene develop normally, without an obvious defect in PCD (Kuida et al., 1995; Li et al., 1995). There are many other members of the Ced-3/ICE family in mammals, however, and any of them could play important roles in PCD (for reviews see Martin and Green, 1995; Kumar, 1995).

There seems to be at least two emerging subfamilies of Ced-3/ICE family proteases, based on their amino acid sequence and the substrates they recognize: those more closely related to ICE, and those more closely related to Ced-3 and CPP32 (Thornberry et al., 1995). ICE, for example, cleaves pro-IL-1 β and is efficiently inhibited in vitro by the tetrapeptide inhibitor Ac-YVAD-CHO (Thornberry et al., 1992), the sequence of which closely resembles the pro-IL-1 β cleavage site (YVHD); CPP32 cleaves the nuclear enzyme poly-(ADP ribose) polymerase (PARP) and is efficiently inhibited in vitro by the tetrapeptide inhibitor Ac-DEVD-CHO (Nicholson et al., 1995), the sequence of which corresponds to the PARP cleavage site (Lazebnik et al., 1994). Two proteases, Mch2 (Fernandes-Alnemri et al., 1995a) and Mch3/ICE-LAP3 (Duan et al., 1996; Fernandes-Alnemri et al., 1995b), have recently been identified that are more similar to CPP32 and Ced-3 than to other family members and are capable of cleaving

PARP in vitro. Although it is still uncertain how many Ced-3/ICE family members there are and which ones are important in PCD in vertebrate cells, there is accumulating evidence that CPP32 and/or one or more of its close relatives are crucially involved.

PARP cleavage is a useful indicator of PCD and of the activation of CPP32 (or its close relatives) in vitro and in vivo. It is cleaved from a 116-kD form to an 85-kD fragment in many examples of PCD (Gu et al., 1995; Kaufmann, 1989; Kaufmann et al., 1993; Tewari et al., 1995). It is cleaved in the same way by cytoplasmic extracts that induce apoptotic changes in isolated nuclei in vitro (Lazebnik et al., 1994), and both the PARP cleavage and nuclear changes are prevented if the extracts are treated with peptide inhibitors that contain the PARP cleavage-site sequence DEVD (Lazebnik et al., 1994; Nicholson et al., 1995). Moreover, when this tetrapeptide sequence was used in an affinity chromatography procedure to purify the protein in the extract that is responsible for cleaving PARP, the purified protein was found to be CPP32 (Nicholson et al., 1995), a previously identified Ced-3/ICE family member (Fernandes-Alnemri et al., 1994).

We show here that STS-induced PCD in many cell types can be suppressed by a cell-permeable tripeptide designed to inhibit members of the Ced-3/ICE family of proteases. Furthermore, we show that CPP32 is activated and PARP is cleaved in STS-induced PCD and that Bcl-2 inhibits both CPP32 cleavage and PCD induced by STS. Most important, we show that intracellular injection of Ac-DEVD-CHO but not Ac-YVAD-CHO blocks STS-induced PCD, indicating that STS-induced PCD in some cells at least requires CPP32, or a CPP32-like member of the Ced-3/ICE family of proteases, but not ICE itself.

Materials and Methods

Animals and Materials

(Balb/c \times C57Bl/6)F1 mice were obtained from Harlan UK Ltd. (Beicester, UK) and maintained in the Animal Facility at University College London. Benzoyloxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone (Z-Val-Ala-Asp(O-Me)-CH₂F; zVAD-fmk) and Benzoyloxycarbonyl-Phe-Ala-fluoromethylketone (zFA-fmk) were from Enzyme Systems Products, Inc. (Dublin, CA). Acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO) was from BAChem (Switzerland). Acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) was a gift from D. Nicholson (Merck-Frosst, Montréal). Stock solutions of peptide inhibitors (25–100 mM) were prepared in dry DMSO and stored in aliquots at -80°C . Working dilutions were made in cell culture medium immediately before use. Calcein-AM and ethidium homodimer were from Molecular Probes-Europe (Amsterdam). STS was a gift from Dr. H. Nakano, Tokyo Research Laboratories, Kyowa Hakko Kogyo Co. Ltd. All other chemicals were from Sigma (UK), unless otherwise indicated.

Cell and Explant Cultures

GM701, an SV40-transformed human fibroblast cell line, GM701 cells stably transfected with the *bcl-2* gene (GM701/*bcl-2* cells; Jacobson et al., 1993), Rat-1, a rat fibroblast cell line, and Rat-1 cells stably transfected with the viral ICE-inhibitor gene *crmA* (Rat-1/*crmA* cells; Miura et al., 1993; a gift of J. Yuan) were maintained in Dulbecco's Modified Eagle Medium containing glucose, Glutamax-1 and sodium pyruvate (DMEM, Gibco), and supplemented with gentamycin (50 $\mu\text{g}/\text{ml}$) and 10% (vol/vol) FCS (Gibco). GM701 cytoplasts were prepared as previously described (Jacobson et al., 1994). Chinese hamster ovary (CHO) cells were maintained in MEM- α medium (Gibco), supplemented with gentamycin and 5% FCS. SKW6.4 cells (Dhein et al., 1992), a human B-cell lymphoma

line, were maintained in RPMI-1640 medium containing 20 mM HEPES and glutamax-1 (Gibco), supplemented with gentamycin and 10% heat-inactivated FCS. Cell lines were maintained at 37°C in either 5% (SKW 6.4 cells) or 10% (GM701 and CHO cells) CO₂ in air.

Organ explant cultures were prepared from freshly isolated mouse organs. Small plugs of tissue were cultured on agarose platforms in 35-mm Falcon bacteriological dishes, containing 0.5 ml DMEM supplemented with 10% FCS, as described in the accompanying paper by Weil et al. (1996). Forepaws were dissected from embryonic day 13.5 (E13.5) mice and cultured as described for organ explants. The two forepaws from each animal were always kept paired, one treated and one untreated, for comparisons between experimental and control groups. In PCD inhibition experiments, zVAD-fmk (100 μM) or vehicle (0.2% DMSO) was added at the beginning of the experiment.

Survival and PCD Assays

For assays on unfixed cells, bisbenzimidazole (HOECHST 33342) was used to stain nuclei, and calcein-AM and ethidium homodimer were used to assess plasma membrane integrity, as previously described (Jacobson et al., 1994). The colorimetric MTT assay (Mosmann, 1983) was performed on cells or cytoplasts grown in 96-well Falcon tissue-culture plates, as previously described (Jacobson et al., 1994).

PCD in fixed cultured cells was assessed by nuclear morphology after staining with propidium iodide (PI) or bisbenzimidazole (HOECHST 33258). For microinjection experiments, cells were grown on poly-D-lysine-coated glass coverslips in a 60-mm Falcon tissue-culture plate. After injection the cells were transferred to a 24-well tissue-culture plate, and, after STS treatment, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for ~1 h at room temperature. After centrifugation to attach floating cells, the cells were postfixed in 50% Carnoy's fixative (acetic-acid/methanol, 1:3) in PBS, followed by 100% Carnoy's fixative (with centrifugation after each step). The cells were then air dried, stained with PI in the presence of RNase (Jacobson et al., 1993), and mounted on glass slides. Cells grown in Falcon 96-well tissue-culture plates were fixed and stained with bisbenzimidazole as follows: cells in 0.1 ml culture medium were fixed by adding 0.1 ml Carnoy's fixative. The plates were centrifuged (1,500 rpm for 5 min), the fix solution and medium were removed and replaced with 0.1 ml fresh fix solution, and the plates were centrifuged again. The fixative was removed and the plates were allowed to dry. Dried plates were stored at room temperature until they were stained by adding 0.1 ml PBS containing 0.1 μg/ml HOECHST 33258. Nuclei were assessed in an Olympus OMT2 inverted fluorescence microscope, using a long working-distance objective, at a final magnification of 400.

PCD in explants was assessed by a combination of the TdT-mediated dUTP-biotin nick end labeling (TUNEL) technique (Gavrieli et al., 1992) and PI staining of cryosections after paraformaldehyde fixation, as described in the accompanying paper by Weil et al. (1996).

PCD in mouse paw explants was assessed by neutral red staining, based on the method in Milligan et al. (1995). To quantitate the regression of interdigital web tissue, web length (WL) was measured in low-power light micrographs of whole fixed paws as follows: WL was taken as the height of the web triangle formed by two digit tips as the base and the proximal end of the interdigital web as the vertex. This distance was normalized to digit length (DL), defined as the average distance between the flanking digit tips and the middle of the second phalanx. The WL/DL ratio was averaged for the two webs flanking the central digit of each paw.

Plotting Inhibition Curves

The % inhibition at inhibitor concentration c was defined as: $\%I(c) = \{(S(c) - \text{MIN}) / (\text{MAX} - \text{MIN})\} \times 100$, where $S(c)$ is the % survival at inhibitor concentration c , MIN is the % survival without inhibitor, MAX is the % survival without PCD inducer or inhibitor. Data (%I vs c) were fit to the sigmoid logistic equation $\%I(c) = 100 \times \{1 / (1 + (IC_{50}/c)^h + 1)\}$, where IC_{50} is the inhibitor IC_{50} and h is the Hill coefficient.

Assays of PARP and CPP32 Cleavage

PARP cleavage was assayed by Western blotting of whole-cell extracts using a monoclonal anti-PARP antibody and the ECL method (Amersham), according to manufacturer's instructions. Cells grown on 60-mm Falcon tissue culture dishes were harvested by brief trypsinization, and both cells and culture supernatant (containing floating dead cells) were collected in 50-ml tubes containing DMEM/10% FCS. The cells were washed once in

1 ml PBS, resuspended at 5×10^5 cells/60 μl in sample buffer (SB; 125 mM Tris, pH 6.95, 15% sucrose, 4% SDS, 10 mM EDTA, 0.001% bromophenol blue, 100 mM dithiothreitol [added fresh]) and boiled for 4 min. The DNA was sheared by trituration through a 23-gauge needle, and the extracts were stored at -20°C until use. Samples (10 μl) were boiled briefly and run on 7.5% SDS-PAGE gels (mini-PROTEAN, BioRad, Richmond, CA), prepared using a modified Laemmli gel recipe. The proteins were transferred from the gel to nitrocellulose, which was then blocked in PBS containing 0.1% Tween-20 and 5% nonfat dry milk (PBST-M), incubated in the monoclonal anti-PARP antibody C-2-10 (Lamarre et al., 1988; a gift of W. Earnshaw, Univ. of Edinburgh, UK and G. Poirier, Université Laval, Quebec; ascites fluid diluted 1:2,000-4,000 in PBST-M), followed by biotinylated sheep anti-mouse Ig (Amersham; diluted 1:3,000 in PBST-M) and then streptavidin HRP (Amersham; diluted 1:1,500 in TBST).

CPP32 cleavage was assayed in extracts of GM701 cells prepared and analyzed as for PARP cleavage, only using a purified mouse anti-human CPP32 monoclonal antibody (Transduction Laboratories, Lexington, KY 1:1,000 in PBST-M). To assay CPP32 cleavage in CHO cells, S100 cytoplasmic extracts were prepared as described by Wang et al. (1995) and diluted 1:1 in 2× concentrated SB. Samples were run on 15% SDS-PAGE gels, transferred to nitrocellulose, incubated with antibodies, and detected by the ECL method, as described above. The primary antibody was a rabbit anti-hamster CPP32 antiserum (M816-4; a gift from J. Goldstein, Univ. of Texas Southwestern Med. Ctr., Dallas, TX diluted 1:2,000 in PBST-M), followed by biotinylated donkey anti-rabbit Ig (Amersham; diluted 1:3,000 in PBST-M), followed by streptavidin-HRP (Amersham; diluted 1:1,500 in PBST+ 0.5% casein). The percentage of apoptotic cells in each sample was determined by taking an aliquot of cells immediately after harvesting; the cells were plated in a 96-well tissue-culture plate, fixed, and stained with bisbenzimidazole, as described above.

Microinjection

The peptide inhibitors Ac-DEVD-CHO and Ac-YVAD-CHO were serially diluted in microinjection buffer (135 mM KCl, 15 mM NaCl, 10 mM Tris-HCl [pH 7.4], 100 μg/ml BSA), containing lysinated FITC-Dextran (Molecular Probes, Eugene, OR; 10,000 mol wt, 8.3 mg/ml). Inhibitor was loaded at the indicated concentrations into a pulled glass capillary tube and injected into cells growing on poly-D-lysine-coated, 13-mm glass coverslips. About 200 cells were injected with one concentration of inhibitor on each coverslip. 30 min to 3 h later, the cells were treated with 2 μM STS for 8-10 h, fixed with paraformaldehyde, and stained with PI, as described above. The coverslips were mounted in Citifluor (University of Kent, Canterbury, UK), sealed with nail varnish, and examined in a Zeiss Axioptan fluorescence microscope. The % inhibition of PCD (%I) was defined as $\{1 - S_{inj}/S_{uninj}\} \times 100$, where S_{inj} and S_{uninj} are the % survival of injected and uninjected cells in the same sample, respectively.

Preimplantation Embryos

Four-cell embryos were obtained from 6-8-wk-old superovulated (Balb-c × C57Bl/6)F1 female mice mated with syngeneic males, as described in the accompanying paper by Weil et al. (1996). The embryos were washed once in M2 medium, transferred to 0.3-ml drops of M16 medium in 35-mm Falcon bacteriological culture dishes, and incubated at 37°C in 5% CO₂. The embryos were treated within 60 min and cell survival was assessed 36 h later using the vital stains HOECHST 33342, ethidium homodimer, and calcein-AM.

Results

zVAD-fmk Inhibits STS-induced PCD in GM701 Cells and Cytoplasts

To determine whether STS-induced PCD in GM701 cells requires Ced-3/ICE-family proteases, we used the irreversible tripeptide inhibitor Z-Val-Ala-Asp(O-Me)-fluoromethylketone (zVAD-fmk), which has been shown to inhibit STS-induced PCD in rat hepatocytes (Cain et al., 1996) and a human monocytic leukemia cell line (Zhu et al., 1995). This inhibitor was designed to enter live cells as the O-methyl ester (of the aspartyl carboxy side-chain)

and to be converted by intracellular esterases into the active inhibitor. Such peptidyl monofluoromethyl ketones are relatively poor nonspecific alkylating agents and have low reactivity toward serine proteinases, but they retain a high degree of reactivity towards cysteine proteinases (Shaw, 1990). Because it lacks an amino acid in the S4 position, zVAD-fmk would be expected to have a broader specificity for Ced-3/ICE family proteases than the tetrapeptide inhibitors of this family such as Ac-DEVD-CHO and Ac-YVAD-CHO.

As shown in Fig. 1 A, zVAD-fmk completely inhibited STS-induced PCD in GM701 cells, when cell viability was assessed by nuclear morphology. The apparent IC_{50} of zVAD-fmk was $\sim 7 \mu M$. zVAD-fmk also completely in-

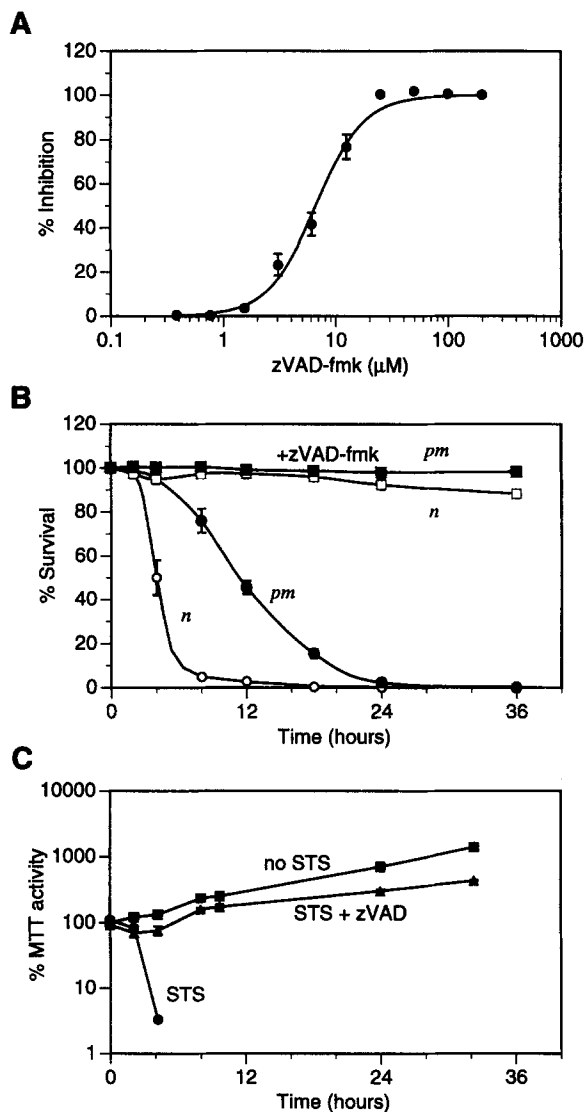


Figure 1. zVAD-fmk inhibits STS-induced PCD in GM701 cells. Cells in a 96-well plate were treated with $2 \mu M$ STS in the presence of various concentrations of zVAD-fmk for 10 h (A) or with $100 \mu M$ zVAD-fmk for various periods of time (B and C). Cell survival was assessed by nuclear morphology after staining with bisbenzimidazole (HOECHST 33258) in A, by nuclear morphology (n) and plasma membrane integrity (pm) in B, and by MTT assay in C. Each point represents the mean \pm SEM from six wells. Each experiment was repeated at least once with the same results.

hibited PCD induced by STS in the presence of $10 \mu g/ml$ CHX (not shown). To determine whether zVAD-fmk-protected cells were also alive by other criteria, we tested their viability by two other assays, MTT and calcein-AM + ethidium homodimer. MTT measures dehydrogenase activity in live cells; calcein-AM labels the cytoplasm of live cells with an intact plasma membrane, whereas ethidium homodimer labels the nucleus of dead cells in which the plasma membrane has broken down. Time course experiments of STS-induced changes in nuclear morphology and plasma membrane integrity (Fig. 1 B), as well as in MTT activity (Fig. 1 C), showed that zVAD-fmk inhibited all of these changes for at least 36 h. Moreover, in cells observed by time-lapse video microscopy, zVAD-fmk prevented the cell shrinkage and loss of intracellular movements normally observed in STS-induced PCD (Jacobson et al., 1994) (not shown). Thus, STS-induced PCD in GM701 cells seems to depend on one or more Ced-3/ICE family proteases.

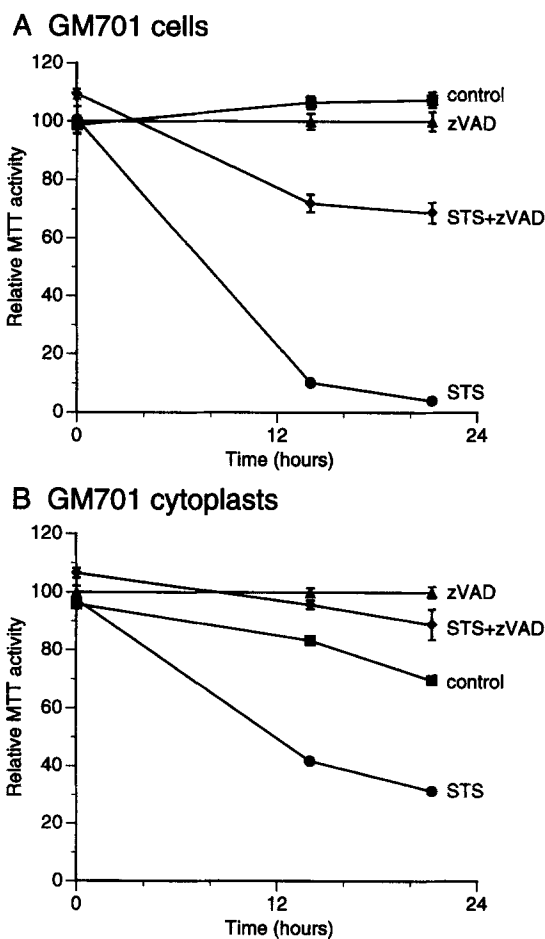


Figure 2. zVAD-fmk inhibits STS-induced PCD in GM701 cells (A) and cytoplasts (B). Cells ($43,000/well$) and cytoplasts ($15,000/well$) in 96-well plates were treated with $100 \mu M$ zVAD-fmk or were left untreated, and $2 \mu M$ STS was added after 20 min. Cell survival was assessed by MTT assay. Each point represents the mean absorbance \pm SEM from six wells (after background subtraction) normalized to the value obtained from wells treated with zVAD-fmk alone for each time point. The relative loss of MTT activity in the (zVAD-fmk + STS)-treated cells (A) reflects the antiproliferative effect of STS, rather than PCD.

We have previously shown that STS can induce PCD in cytoplasts. As shown in Fig. 2, cytoplasts prepared from GM701 cells died (as assessed by loss of MTT activity) after treatment with STS, just like the nucleated cells, and zVAD-fmk inhibited the STS-induced loss of MTT activity in both the cytoplasts and nucleated cells. Therefore, at least one of the relevant zVAD-fmk-inhibitable proteases is present in the cytoplasm and can be activated in the absence of a nucleus.

zVAD-fmk Does Not Inhibit Cell Proliferation, Even in the Presence of a PCD-inducing Stimulus

If zVAD-fmk is to be useful as a tool, in studying the role of PCD in developmental processes, for example, it is important that it not inhibit other cell functions such as cell proliferation. To test whether cells protected from PCD by zVAD-fmk are still able to proliferate, we had to turn to an alternative inducer of PCD, as STS inhibits cell proliferation when used at the high concentrations required to induce PCD (Abe et al., 1991; Bruno et al., 1992; Crissman et al., 1991), even when cells are protected from PCD by zVAD-fmk (see Fig. 2 A) or *bcl-2* overexpression (not shown). As shown in Fig. 3 A, zVAD-fmk completely inhibited anti-APO-1/Fas-antibody induced PCD (as assessed by nuclear morphology) in the human B-cell lymphoma line SKW 6.4 and was about ten times more potent in doing so than in inhibiting STS-induced PCD in these cells. A dipeptide-fmk (zFA-fmk), designed as a cathepsin B inhibitor (Rasnick, 1985), which would not be expected to bind to Ced-3/ICE proteases, had no effect, even at 300 μ M. As shown in Fig. 3 B, 100 μ M zVAD-fmk had no apparent effect on the growth rate of SKW 6.4 cells for 48 h, even in the presence of anti-APO-1/Fas antibodies (in this experiment, anti-APO-1/Fas, in the absence of inhibitor, was not simply cytostatic, as most of the trypan blue-excluding cells, defined as viable, appeared shrunken and were probably apoptotic). Therefore zVAD-fmk seems not to inhibit cell proliferation, and, in some cases at least, cells can still proliferate in the presence of a PCD-inducing stimulus, if zVAD-fmk is protecting them from PCD.

STS-induced Activation of Ced-3/ICE-Family Proteases Occurs with a 2–3-h Delay

To determine when the Ced-3/ICE-family proteases are activated after STS treatment, we treated GM701 cells with zVAD-fmk at various times before or after adding 2 μ M STS. As shown in Fig. 4, cells treated with STS without zVAD-fmk were not apoptotic at 3 h but were all apoptotic by 4 h; cotreatment with 10 μ g/ml cycloheximide (CHX) did not alter the kinetics of death (not shown). By contrast, when cells were treated with zVAD-fmk as late as 2 h after the addition of STS, they were still nearly all alive after 10 h; treatment with inhibitor 4 h after STS addition, however, failed to protect the cells, while addition 3 h after STS addition protected ~40% of them. These findings suggest that the relevant Ced-3/ICE-family proteases are activated just before the onset of apoptosis, and when these proteases become active, cell death occurs quickly.

PARP and CPP32 Are Cleaved in STS-induced PCD

To determine whether CPP32, or a CPP32-like protease, is

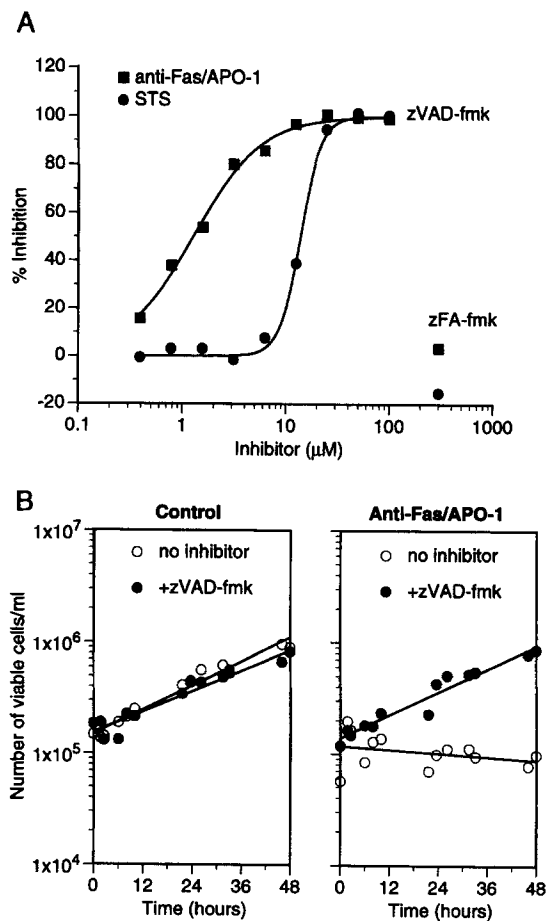


Figure 3. zVAD-fmk inhibits anti-Fas/APO-1-induced PCD in SKW6.4 cells (A), but not their proliferation (B). In A, cells in a 96-well plate were treated with zVAD-fmk or the control peptide zFA-fmk 30 min before the addition of anti-Fas/APO-1 antibody (50 ng/ml) or STS (2 μ M) for 7 h; viability was assessed by nuclear morphology after fixation. The % inhibition data shown represent % survival normalized to the maximum (no inducer) and minimum (no inhibitor) values. Survival in the absence of inhibitor was 12% for anti-Fas/APO-1-treated cells, and 20% for STS-treated cells; survival in the absence of inducer was 93%. In B, cells in a 24-well plate were treated with anti-Fas/APO-1 antibody (50 ng/ml), with or without zVAD-fmk (100 μ M), and aliquots of cells were sampled at various times; cell viability was assessed by trypan blue exclusion. Doubling times ($h \pm$ SEM) obtained from fitting the data to a single exponential were no inhibitor, 19.8 ± 1.4 ; zVAD-fmk, 21.9 ± 1.6 ; anti-Fas/APO-1 + zVAD-fmk, 19.4 ± 1.5 . Anti-Fas/APO-1 alone was not simply cytostatic; although most of the cells in these cultures excluded trypan blue, by 6 h most were shrunken and probably apoptotic (as in A). The experiments shown in A and B were repeated once with the same results.

activated during STS-induced PCD, we first looked for PARP cleavage, using Western blotting of extracts of STS-treated GM701 cells, as PARP is a known substrate for CPP32 (Nicholson et al., 1995). As shown in Fig. 5, STS treatment induced PARP cleavage and produced an ~85-kD fragment, as expected for cleavage by CPP32. PARP cleavage occurred with the same time course (Fig. 5 A) as the appearance of pyknotic nuclei (see Fig. 4) and did not occur in cells protected with zVAD-fmk (Fig. 5 B).

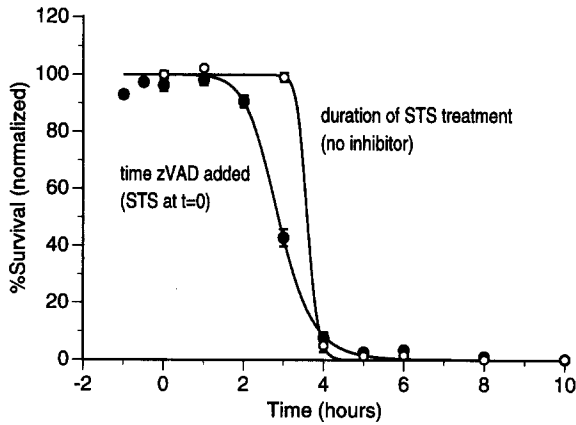


Figure 4. The effect of delayed addition of zVAD-fmk. GM701 cells in a 96-well plate were treated either with 2 μ M STS alone for the times indicated (*open circles*) or with 2 μ M STS for 10 h, with 100 μ M zVAD-fmk added at various times relative to the start of STS treatment (*closed circles*). The drugs were added in a staggered manner so that all cells were fixed at the same time at the end of the experiment. Cell survival was assessed as in Fig. 1 A. Each point represents the mean \pm SEM of results from six wells. Survival values were normalized to the survival with STS at time = 0 without inhibitor, which was 93%.

We next examined whether CPP32 itself was cleaved in STS-treated cells. As shown in Fig. 6 A, the 32-kD proform of CPP32 was cleaved in STS-treated GM701 cells with the same time course as PARP cleavage (cf. Fig. 5 A), and this cleavage was markedly inhibited in cells protected by either zVAD-fmk (Fig. 6 B) or by overexpression of Bcl-2 (Fig. 6 C). As the antibody used to detect human CPP32 did not detect the cleavage products, we used a hamster-specific antiserum raised against an NH₂-terminal peptide of the p20 subunit of hamster CPP32 (Wang et al., 1996) to study extracts of STS-treated Chinese hamster ovary (CHO) cells. As shown in Fig. 6 D, pro-CPP32 was cleaved in these cells to a 17-kD product with a time course identical to the occurrence of PCD (determined in the same experiment and shown at the bottom of Fig. 6

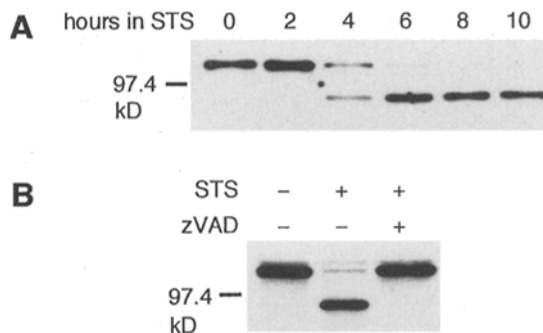


Figure 5. PARP cleavage in STS-treated GM701 cells, assessed by Western blotting. (A) Time course in 2 μ M STS. (B) Effect of 100 μ M zVAD-fmk on cells treated with 2 μ M STS. Cell lysates were run on 7.5% SDS-PAGE gels, transferred to nitrocellulose, incubated in a monoclonal anti-PARP antibody and visualized by ECL, as described in Materials and Methods.

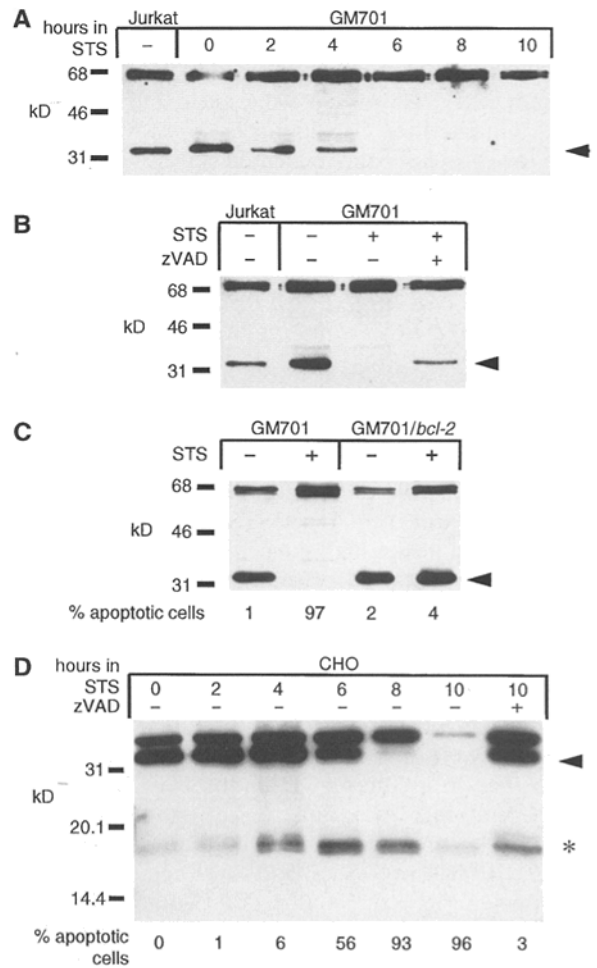


Figure 6. CPP32 cleavage in STS-treated GM701 and CHO cells, assessed by Western blotting. In A and B STS-treated GM701 cell lysates from the experiment shown in Fig. 5 were run on 15% SDS-PAGE gels, and Western blots were performed with a monoclonal anti-human CPP32 antibody, as described in Materials and Methods. A time course of CPP32 cleavage following 2 μ M STS treatment is shown in A, and inhibition by 100 μ M zVAD-fmk is shown in B; in both, a whole cell lysate from Jurkat cells (supplied by Transduction Laboratories) was used as a positive control. In C, S100 cytoplasmic fractions of GM701 and GM701/bcl-2 cells, either untreated or treated with 2 μ M STS for 9 h, were analyzed as in A and B. In D, S100 cytoplasmic fractions of CHO cells treated with 2 μ M STS were analyzed by Western blotting, as described for the GM701 cell lysates, using a rabbit anti-hamster CPP32 antiserum. 32-kD pro-CPP32 is indicated by arrowheads, and the 17-kD cleavage product in D by an asterisk. The proportion of apoptotic cells in each sample in C and D is shown at the bottom of each lane; the experiment in C was repeated twice with the same results. The additional bands seen at \sim 68 kD in A-C and $>$ 32 kD in D are probably not CPP32-related, as they are unaffected by the experimental conditions; they show that equivalent amounts of protein were loaded in each lane.

D), and this cleavage was greatly inhibited in cells protected with zVAD-fmk (right-hand lane in Fig. 6 D). Taken together, these experiments suggest that CPP32 is activated in STS-induced PCD, at least in GM701 and CHO cells.

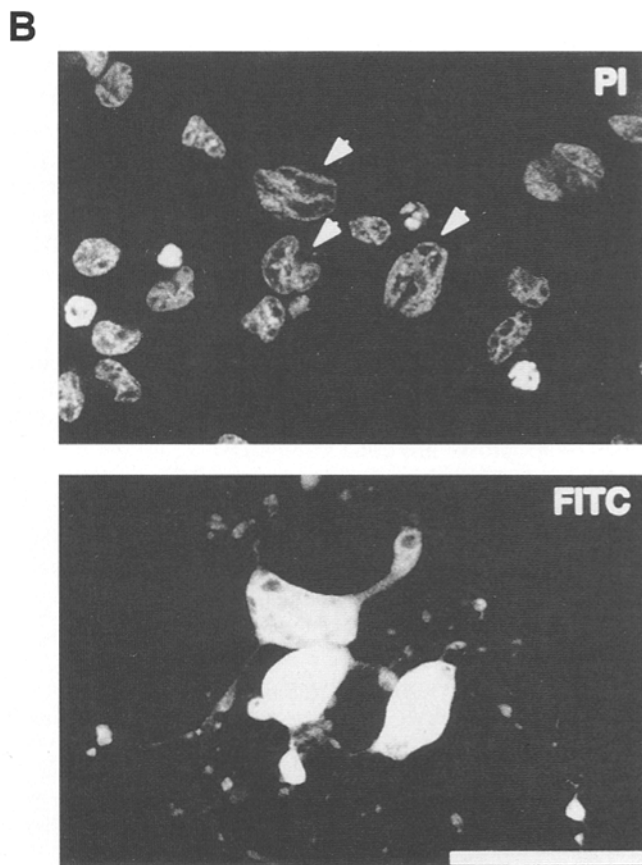
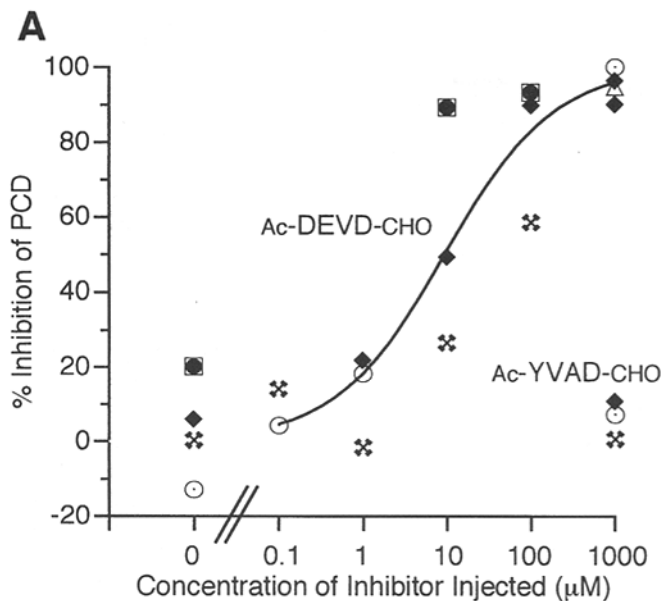


Figure 7. Microinjection of Ac-DEVD-CHO, but not Ac-YVAD-CHO, inhibits STS-induced PCD in GM701 cells. Cells on coverslips were injected with Ac-DEVD-CHO, Ac-YVAD-CHO, or buffer alone, along with FITC-Dextran to identify the injected cells. After injection, the cells were treated with 2 μM STS for 8–10 h, and the proportions of injected and uninjected cells with pyknotic nuclei were determined following paraformaldehyde fixation and staining with PI (A). Each point represents cells on a separate coverslip, and each type of symbol represents a separate experiment. Percent inhibition of PCD was calculated as described in Materials and Methods, and represents data normalized to the

STS-induced PCD Depends on CPP32 or a CPP32-like Protease(s)

The above experiments indicated that CPP32 is cleaved in STS-induced PCD, but they did not indicate whether this cleavage is required for STS-induced PCD. To determine whether CPP32, or a CPP32-like protease, is required for STS-induced PCD, we microinjected the tetrapeptide CPP32 inhibitor Ac-DEVD-CHO (along with FITC-Dextran to mark the injected cells) into cultured GM701 cells and then treated the cells with STS. The cells were fixed 8–10 h later and assayed for PCD by analyzing nuclear morphology. As shown in Fig. 7, cells microinjected with Ac-DEVD-CHO were protected against STS-induced PCD: whereas, on average, $88 \pm 7\%$ of the uninjected and $79 \pm 9\%$ of the vehicle-injected cells were apoptotic, less than 10% of the cells injected with the highest concentrations of the inhibitory peptide were apoptotic. The apparent IC_{50} for Ac-DEVD-CHO was 9 μM in the injection pipette. Assuming an injection volume of $\sim 1\text{--}10\%$ of total cell volume, this corresponds to an intracellular concentration of $\sim 0.1\text{--}1 \mu\text{M}$. Even when 1 mM (10–100 μM intracellular) Ac-YVAD-CHO was injected, there was no protective effect, suggesting that ICE itself was not required for STS-induced PCD.

To test further whether ICE is required for STS-induced PCD, we examined a stably transfected line of Rat-1 cells that overexpresses the Cowpox virus protein CrmA (Rat-1/*crmA* cells, Miura et al., 1993), which is a potent inhibitor of ICE (Ray et al., 1992). When overexpressed, CrmA has been shown to inhibit anti-Fas/APO-1 and $\text{TNF}\alpha$ -induced PCD (Enari et al., 1995b; Los et al., 1995; Miura et al., 1995; Tewari and Dixit, 1995). Rat-1/*crmA* cells, however, were just as sensitive to STS-induced PCD as control Rat-1 cells (not shown), consistent with the conclusion that STS-induced PCD does not depend on ICE, at least in Rat-1 and GM701 cells.

zVAD-fmk Inhibits STS-induced PCD in Many Cell Types, but Not Blastomeres

High concentrations of STS, in the presence of cycloheximide to inhibit protein synthesis, induce PCD in all of the many nucleated mammalian cell types we have examined. To test whether Ced-3/ICE family proteases are involved in most of these cases, we studied two types of primary culture systems that we had studied previously, explants of newborn mouse organs (see accompanying paper by Weil et al. [1996]), and dissociated cells from E13 mouse embryos (minus the head and liver) (Ishizaki et al., 1995). As shown in Fig. 8, *zVAD-fmk* inhibited (STS + CHX)-induced PCD in explant cultures of neonatal skin (Fig. 8

survival of uninjected cells (S_{uninj}) on each coverslip ($S_{\text{uninj}} = 14 \pm 16\%$, mean \pm SD for all 22 data points). The apparent IC_{50} derived from fitting the data to sigmoid logistic equation was $9.4 \pm 1.9 \mu\text{M}$ (see Materials and Methods). A confocal micrograph of STS-treated GM701 cells microinjected with 1 mM Ac-DEVD-CHO is shown in B, where the PI stained nuclei are shown in the top panel and the FITC-Dextran-labeled cells in the bottom panel. The non-apoptotic nuclei of injected cells are indicated by arrowheads. Bar, 50 μm .

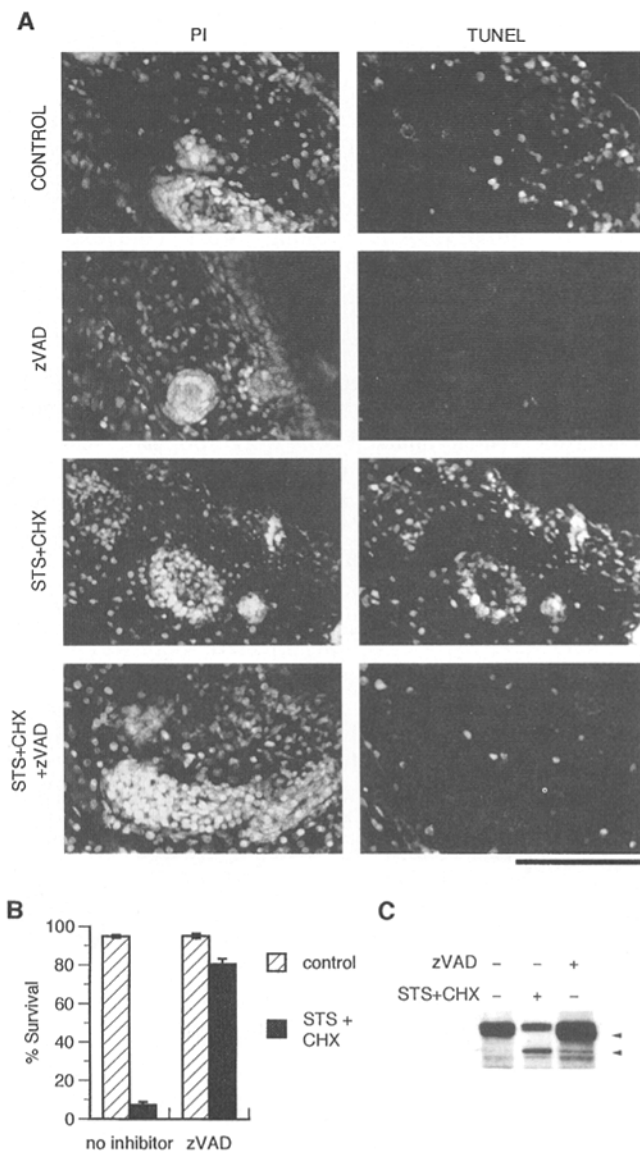


Figure 8. Cell survival and PARP cleavage in perinatal mouse cells. (A) PI- and TUNEL-labeled sections of neonatal skin explants treated with vehicle (0.2% DMSO), zVAD-fmk (100 μ M), STS (10 μ M) + CHX (10 μ g/ml), or STS + CHX + zVAD-fmk. (B) Survival of 1-d-old cultures of E13 mouse embryo cells, either untreated or treated with STS (1 μ M) + CHX (10 μ g/ml) with or without zVAD-fmk (100 μ M) for 24 h. (C) PARP cleavage in E13 mouse embryo cells from the same experiment as shown in B. In C, full-length PARP and the larger cleaved form are each indicated by arrowheads. Bar in (A) 100 μ m.

A), as well as in freshly prepared cultures of E13 embryo cells (Fig. 8 B). Moreover, all of the detectable PARP was cleaved in lysates prepared from the (STS + CHX)-treated E13 cells, and this cleavage was greatly inhibited in cells that were protected by zVAD-fmk (Fig. 8 C). zVAD-fmk also inhibited (STS + CHX)-induced PCD in explants of neonatal kidney, heart, and lung, and adult lung, kidney, and ear pinna (not shown). These findings suggest that one or more Ced-3/ICE-family proteases are required for (STS + CHX)-induced PCD in most nucleated mammalian cells.

Not all cells, however, can be protected from STS-induced PCD. When four-cell mouse embryos were treated with 10 μ M STS (with or without CHX) for 36 h, the cells died with the morphological features of apoptosis (see accompanying paper by Weil et al. [1996]), even in the presence of 100 μ M zVAD-fmk (Table I).

zVAD-fmk Inhibits Interdigital PCD in Developing Mouse Paws

To determine whether Ced-3/ICE-family proteases are involved in the PCDs that occur during normal development (Glucksmann, 1951), we removed the forepaws from E13.5 mouse embryos and cultured them for 26 h on agarose platforms. During this culture period, PCD in the interdigital spaces separated the developing digits, just as it normally does in vivo. Treatment with zVAD-fmk greatly inhibited this cell death (assayed by neutral red staining after 5 h in culture) and largely prevented the removal of the interdigital webs, assessed after 26 h in culture (Fig. 9).

Discussion

STS-induced PCD has been a useful model for studying some of the features of the basic core machinery of PCD. Its main advantage as a model is that it apparently can be used with any nucleated mammalian cell. One of the conclusions drawn from the STS model is that nucleated mammalian cells constitutively express all the proteins required to undergo PCD. The reliability of this conclusion depends on the evidence that STS-induced, and (STS + CHX)-induced, cell deaths are bona fide PCDs. Our findings and those of others (Cain et al., 1996; Mashima et al., 1995; Zhu et al., 1995), that peptide inhibitors of the Ced-3/ICE family of proteases can inhibit these deaths in a large variety of cell types, provide the strongest evidence that these deaths, which display the morphological features of apoptosis, are bona fide PCDs.

Another conclusion drawn from the STS model is that PCD does not require a nucleus and is controlled from the cytoplasm: anucleate cytoplasts prepared from GM701 cells were shown to die with the same kinetics and morphological features when treated with STS as did the par-

Table I. Effect of zVAD-fmk on STS-induced and (STS+CHX)-induced PCD in Four-Cell Mouse Blastomeres

Treatment	Live blastomeres/total blastomeres	
	EXPT 1 (blastomeres)	EXPT 2 (whole embryos)
	%	
Control	(>95)*	(>95)*
zVAD-fmk	104/115 (90)	(>95)*
STS	ND	0/79 (0)
STS+zVAD-fmk	ND	0/65 (0)
STS+CHX	0.77 (0)	0/109 (0)
STS+CHX+zVAD-fmk	0/67 (0)	0/146 (0)

Embryos or dissociated blastomeres were cultured for 36 h. STS was used at 10 μ M, CHX at 10 μ g/ml, and zVAD-fmk at 100 μ M. Cell viability was assessed by calcein-AM and ethidium homodimer staining. Cell division did not occur in STS, but it did so in controls and when zVAD-fmk was used alone, which made cell counting difficult so that the total numbers of cells in these cases (indicated with an asterisk) were estimates. ND, not done.

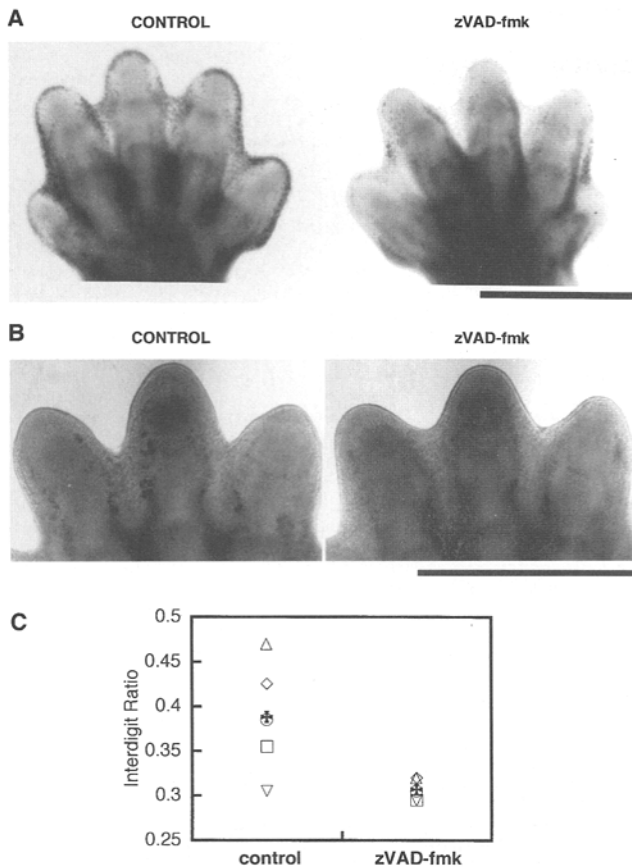


Figure 9. zVAD-fmk inhibits normal interdigital PCD in explant cultures of developing paws. (A) E13.5 mouse forepaws stained with neutral red after 5 h in culture with (*right paw*) or without (*left paw*) 100 μ M zVAD-fmk. (B) E13.5 mouse forepaws after 26 h in culture with (*right paw*) or without (*left paw*) 100 μ M zVAD-fmk, showing zVAD-fmk inhibition of the loss of interdigital webs. (C) Quantitation of inhibition of interdigital web loss by zVAD-fmk. Each pair of paws from one animal (one control, one zVAD-fmk treated) is indicated with the same symbol; the average interdigital ratio in each condition is indicated with a cross. The interdigital ratios in the zVAD-fmk-treated paws were significantly different from untreated controls ($P = 0.0253$, paired t test). A–C were each from separate experiments. In all three experiments, zVAD-fmk inhibited the loss of interdigital webs. Bar, 1 mm.

ent cells (Jacobson et al., 1994). Again, the reliability of this conclusion depends on the evidence that the death of the cytoplasm is bona fide PCD. The present finding that STS-induced death of cytoplasm is inhibited by zVAD-fmk strongly supports the claim that the STS-treated cytoplasm die by PCD and suggests that at least one of the relevant Ced-3/ICE family members is present in the cytoplasm and can be activated in the absence of a nucleus. The conclusion that the death program is orchestrated from the cytoplasm is also supported by findings of others that Fas-dependent PCD can occur in cytoplasm (Nakajima et al., 1995; Schulze-Osthoff et al., 1994) and that cytoplasmic extracts can induce apoptotic changes in isolated nuclei in vitro (Enari et al., 1995a; Lazebnik et al., 1993, 1994; Martin et al., 1995; Newmeyer et al., 1994).

We previously studied the kinetics of STS-induced PCD in GM701 cells, using 1 μ M STS. We found that the first cells started dying after \sim 2–4 h, and most died by \sim 8–12 h (Jacobson et al., 1993, 1994). In the present study we used a higher concentration of STS (2 μ M) on GM701 cells, and, although there was still a lag of 2–3 h, most of the cells died by 4 h (see Fig. 4). Although the time course of PCD is somewhat variable from experiment to experiment and from cell line to cell line, there is always a lag phase of at least 2 h, which is unaffected by cotreatment with CHX. It is not clear what happens in the 2-h lag period to induce PCD, although it presumably involves the inhibition of one or more protein kinases. Whatever the mechanism of induction, it is clear that the required Ced-3/ICE family proteases that are inhibited by zVAD-fmk are not activated until the end of the lag period, as zVAD-fmk is still an effective inhibitor even when added up to 2 h after STS is added, just before the first appearance of PCD (see Fig. 4). Thus, once these proteases are activated, PCD follows very rapidly.

The finding that zVAD-fmk blocks (STS + CHX)-induced PCD in a wide variety of cells, including GM701 cells, a mixture of mouse embryo cells, and explant cultures of various newborn and adult mouse organs, suggests that Ced-3/ICE-family proteases are required for (STS + CHX)-induced PCD in the majority of mammalian cell types. However, zVAD-fmk does not inhibit STS-induced, or (STS + CHX)-induced, PCD in four-cell-stage mouse blastomeres, suggesting that zVAD-fmk cannot be used as a universal inhibitor of PCD. It is possible that STS-induced death of blastomeres does not involve Ced-3/ICE family members, or involves family members that are resistant to zVAD-fmk. Alternative explanations seem more likely to us: blastomeres may handle the drug differently, for example, either excluding it, pumping it out, or inactivating it in some way.

Which of the Ced-3/ICE family members are involved in STS-induced PCD? Inhibition by zVAD-fmk provides a few clues, as it is unclear which family members zVAD-fmk inhibits, especially when used on live cells. We provide two lines of evidence that suggest that ICE itself is not involved. First, CrmA, which binds to and inactivates ICE (Ray et al., 1992), does not inhibit STS-induced PCD in Rat-1 cells. Interestingly, CrmA can inhibit anti-Fas/APO-1-induced PCD and TNF α -induced PCD in cultured cells (Enari et al., 1995b; Los et al., 1995; Miura et al., 1995; Tewari et al., 1995) and can inhibit CPP32-associated PARP cleavage in vitro (Tewari et al., 1995). Presumably CrmA inhibits ICE and/or other related proteases that are involved in a proteolytic cascade upstream of CPP32 activation and are required for anti-Fas/APO-1- and TNF α -, but not STS-triggered PCD.

The second line of evidence is more direct: microinjection of Ac-YVAD-CHO, a potent tetrapeptide inhibitor of ICE (Thornberry et al., 1992), does not inhibit STS-induced PCD in GM701 cells. By contrast, several lines of evidence suggest that CPP32 and perhaps other closely related family members are involved in STS-induced PCD. First, STS induces PARP cleavage in both GM701 cells and E13 mouse embryo cells with the same time course as it induces PCD, and the cleavage product resembles that produced when CPP32 cleaves PARP in vitro. Second, STS

induces pro-CPP32 cleavage in both GM701 cells and CHO cells, with the same time course as it induces PCD, and the 17-kD product found when pro-CPP32 is activated by cleavage is found in STS-treated CHO cells, indicating that CPP32 is itself activated during STS-induced PCD in these cells. Wang et al. (1996) and Chinnaiyan et al. (1996) observed CPP32 cleavage in STS-induced PCD, and Wang et al. (1996) showed that activated CPP32 cleaves sterol regulatory element-binding proteins (SREBPs) in PCD, identifying SREBPs as additional targets of active CPP32. Our finding that zVAD-fmk blocks the STS-induced activating cleavage of CPP32 suggests that zVAD-fmk either inhibits at least one Ced-3/ICE family member, which is not ICE, upstream of CPP32, or inhibits CPP32 from cleaving itself. Third, CPP32 is not activated in cells that are protected from PCD by overexpression of Bcl-2. This suggests that Bcl-2 acts upstream of the activation of at least some of the Ced-3/ICE family proteases. Fourth, and most important, microinjection of the tetrapeptide Ac-DEVD-CHO, which is a more potent inhibitor of CPP32 than of ICE, blocks STS-induced PCD in GM701 cells. This finding suggests that not only is CPP32 activated, but the activation of CPP32 and/or a CPP32-like protease, such as Mch2 and Mch3/ICE-LAP3, is required for STS-induced PCD in these cells.

The discovery that Ced-3 is homologous to ICE (Yuan et al., 1993) provided the critical evidence that linked this family of proteases to PCD. This linkage may at last provide the beginnings of a molecular definition and understanding of PCD. Inhibitors of the Ced-3/ICE family such as zVAD-fmk may, for example, provide a specific way to inhibit PCD and thereby to determine the function of PCD in various developmental processes in vertebrates. It was with this in mind that we tested the ability of zVAD-fmk to inhibit PCD in a situation where the role of cell death is known, in the interdigital spaces during digit development. The finding that zVAD-fmk inhibits both interdigital PCD and the removal of the interdigital web during digit formation in explant cultures of developing mouse paws is encouraging. In independent studies, Milligan et al. (1995) showed that the peptide inhibitor Ac-YVAD-CHO suppressed interdigital PCD in the developing chick limb bud in ovo, although they did not test whether the inhibitor blocked the removal of the interdigital webs. It will now be of interest to test these inhibitors in situations where the role of PCD is unknown.

In summary, our findings help to establish STS-induced PCD as a legitimate model of PCD and add to the accumulating evidence that points to CPP32 and/or its close relatives as crucial components of the death machinery in mammalian cells. Our findings also demonstrate the usefulness of cell-permeable inhibitors of the Ced-3/ICE family of proteases for studying PCD in intact cells and tissues.

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