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Caspase-3/CPP32-like activity is not sufficient to mediate apoptosis in an IL-2 dependent T cell line

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CTLL cells undergo apoptosis when cultured in the absence of IL-2. The IL-1β-converting-enzyme (ICE)/caspase family has been implicated as an integral component of some forms of apoptosis. Numerous members of the caspase family have been identified, and it appears as if caspase-3/CPP32 plays a critical role. Previously we demonstrated that ICE/caspase-1 expression increases in CTLL cells during apoptosis; however, inhibition of ICE activity did not abrogate apoptotic death. The purpose of this report is to determine if other members of the caspase family are involved in T cell apoptosis induced by growth factor starvation. We show that cytosolic CPP32-like activity, as measured by the cleavage of DEVD-pNA and poly(ADP-ribose) polymerase (PARP), increases during apoptosis following growth factor deprivation. Cytosolic CPP32-like activity is inhibited in cells treated with the broad spectrum ICE family inhibitor boc-aspartyl(OMe)-fluoromethylketone (D-FMK) and by VAD-FMK and DEVD-FMK which have greater specificity for CPP32-like ICE homologs; however, only the broad spectrum ICE inhibitor D-FMK inhibited apoptosis. Our results suggest that apoptosis induced by growth factor deprivation involves the caspase family, but increased CPP32-like activity is not sufficient to mediate apoptosis induced by IL-2 starvation.

Key words: Caspase; fluoromethylketones; CPP32; PARP; T cells.

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Introduction

Apoptosis or programmed cell death (PCD) is a fundamental process of immune system development and homeostasis. During lymphocyte differentiation, thymocytes and pre-B-cells are depleted by apoptosis following receptor gene rearrangements which are faulty or result in receptors that may be autoreactive. Peripheral T-lymphocytes also undergo apoptotic death following either T cell receptor stimulation or a decrease in cytokine production after antigen clearance, thereby removing antigen-specific effector cells that could contribute to inflammation or autoimmunity. Indeed, PCD dysfunction in the immune system can lead to disease. For example, in AIDS, dysregulated apoptosis occurs in non-HIV infected T cells, while in lupus erythematosus, a defect in the Fas/Apo-1 gene results in aberrant lymphocyte apoptotic death.[3–5]

Crucial insights into the mechanisms of apoptosis and PCD have been provided by genetic analysis of the nematode *Caenorhabditis elegans*. Evidence implicating the IL-1β-converting-enzyme (ICE)/caspase family in mammalian apoptosis and PCD is based on the following: (i) structural homology to *ced-3*; (ii) apoptosis induced by caspase-1/ICE homolog overexpression; (iii) cleavage of structural and regulatory proteins during apoptosis induced by caspases; (iv) inhibition of apoptosis by the caspase family-specific anti-apoptotic viral genes *crmA* and *p35*; and (v) inhibition of apoptosis with pharmacological inhibitors specific for the caspase family.[24] Determining which caspase family mem-

bers are required for apoptosis has been difficult to elucidate, because several ICE homologs have been identified and specific inhibitors for the homologs have not yet been produced. The overall evidence suggests that caspase-3/CPP32 proteases play a key role in apoptosis.

Evidence implicating CPP32 as an important component in apoptosis exists in its phylogenetic relationship with other caspase family members along with biochemical and pharmacologic evidence. Members of the caspase family can be clustered into three subfamilies separated by structural differences: those that are most similar to caspase-1/ICE (caspase-4/Ich-2/TX/ICE_{rel-II}, caspase-11/Ich-3, caspase-5/TY/$ICE_{rel-III}$), and those similar to CED3 (caspase-3/CPP32, caspase-6/Mch2, caspase-9/Mch6/ICE-LAP6, caspase-7/Mch3/ICE-LAP3/CMH-1/SCA-2) and the most distinct family consisting of caspase-2/Ich-1/Nedd2.[10,21,25–33] CPP32 is the most highly related member of the ICE protease family to CED3, which is essential for PCD in *C. elegans*, including very high conservation of amino acids that confer substrate specificity.[18] CPP32 can also cleave at least six substrates at the initiation of apoptosis presumed to be critical in the apoptotic process: poly(ADP-ribose) polymerase (PARP), SREBPs, U1-70K, non-erythroid α-spectrin, DNA-PK_{cs} and D4-GDI.[24,34–38] Furthermore, CPP32 can be activated by the CTL-derived granzyme B suggesting a mechanism by which granzyme B launches a suicide response in CTL targets.[39] Finally, inhibitors of CPP32 inhibit apoptosis in several mammalian systems, whereas similar concentrations of ICE inhibitors do not halt the apoptotic process.[16,17,19,23,24] Overall, CPP32 appears to be an important caspase family protease in apoptosis; however, closely related family members such as caspase-6/Mch2 and caspase-7/Mch-3 can not be ruled out, particularly since they may cleave the same putative substrates.[30]

In this report, we examined the role of the caspase family in apoptosis in the lymphokine-dependent CTLL T cell line. Apoptosis in CTLL cells can be induced by IL-2 starvation and is inhibited by *bcl-2* overexpression or by protein synthesis inhibition.[40,41] Although ICE expression increases in CTLL cells undergoing apoptosis, ICE proteolytic activity is not required for apoptosis in these cells.[42] The objective of this report is to determine if the caspase family is required for apoptosis in CTLL cells. We examined the role of the caspase family during apoptosis in CTLL cells using numerous pharmacologic inhibitors of the caspase family, each with varying specificities to the caspase members. We also examined cytosolic extracts of cells undergoing apoptosis for CPP32-like activity, and determined the effect of the pharmacologic inhibitors on CPP32-like activity. We report here that CPP32-like activity increases in CTLL cells undergoing apoptosis induced by IL-2 starvation, and the increase in activity is most prevalent at the time when the cells can no longer be rescued with the growth factor IL-2. Moreover, apoptosis is abrogated with a broad spectrum inhibitor of the caspase family, but more specific inhibitors of CPP32-like activity do not inhibit apoptosis.

Materials and methods

Cell culture and reagents

CTLL cells (American Type Culture Collection, Rockville, MD, USA) were maintained in complete RPMI 1640 medium (cRPMI) containing 10% FCS, 2 mM L-glutamine, 2 mM sodium pyruvate, 5.5×10^{-5} M 2-ME, 50 μg/ml gentamicin sulfate and 50 U/ml recombinant human IL-2 (Life Technologies, Gaithersburg, MD, USA). Boc-aspartyl (OMe)-fluoromethylketone (D-FMK), benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl-ketone (VAD-FMK), benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (DEVD-FMK), benzyloxycarbonyl-Phe-Ala-fluoromethylketone (FA-FMK), boc-Thr-fluoromethylketone (T-FMK) were obtained from Enzyme Systems Products (Dublin, CA). Acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO), acetyl-Tyr-Val-Ala-Asp-aldehyde (YVAD-CHO), acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA), acetyl-Tyr-Val-Ala-Asp-*p*-nitroanilide (YVAD-pNA), Suc-Leu-Tyr-7-amino-4-methylcoumarin (LY-AMC), Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (LLVY-AMC) were obtained from Bachem Biosciences (King of Prussia, PA, USA). All fluoromethylketones (FMK), aldehydes (CHO), *p*-nitroanilides (pNA) and amino methylcoumarins (AMC) were diluted to 50 mM in sterile, cell culture grade DMSO (Sigma Chemical Co, St. Louis, MO, USA) and stored at -20°C

in desiccant. Cycloheximide (CHX, Sigma) and etoposide (Sigma) were freshly prepared before each experiment. Sodium azide (Sigma) was stored at room temperature as a 10% stock solution.

Caspase and calpain activity assays

Cytosolic extracts were collected from CTLL cells as described previously.[43] Briefly, cells were washed twice after culture in PBS and lysed in S-buffer (50 mM NaCl, 2 mM $MgCl_2$, 40 mM β-glycerophosphate, 5 mM EGTA, 10 mM HEPES, pH 7.5) by freezing and thawing four-times [1×10^8 cells per ml lysis buffer]. The extracts were stored at -70°C.

Cytosolic caspase-3/CPP32-like and caspase-1/ICE-like protease activities were measured in spectrophotometric assays using *p*-nitroanilide substrates in 96-well plates.[44] The ICE assay was performed with Ac-YVAD-pNA substrate [50 µM] and 11–200 µg cytosolic extract; the CPP32 assay was performed with Ac-DEVD-pNA substrate [100 µM] and 11–33 µg cytosolic extract. The reaction buffer for both enzymes contained 100 mM HEPES, pH 7.5, 0.5 mM EDTA, 20% glycerol and 0.05% BSA (HGE + BSA buffer) in a total volume of 100 µl per well. DTT [10 mM] was included in the ICE assay. The plates were incubated for 5 min at 30°C before kinetic analysis of activity at 405 nm (SOFTmax, version 2.35, Molecular Devices Corporation, Sunnyvale, CA). The data are presented as velocity in mOD/min. In some experiments rcaspase-1/rICE [50 nM] or rcaspase-3/rCPP32 [10 nM] was used in place of the cytosolic extracts. Ac-YVAD-CHO and Ac-DEVD-CHO were used to inhibit rICE and rCPP32, respectively, and the cleavage of pNA substrates by the cytosolic extracts. Recombinant ICE and rCPP32 were produced and purified as described previously.[44]

Cytosolic CPP32-like activity was also measured by cleavage of ^{35}S-labelled PARP.[43] Cytosolic extracts (5 µg) were incubated with one µl of ^{35}S-labelled PARP in 20 µl of HGE + BSA buffer for 10 min at 30°C. The reaction mixture was diluted in SDS-PAGE buffer containing 2-mercaptoethanol and separated by electrophoresis on 8% Tris-glycine gels (Novex, San Diego, CA, USA). The gels were treated with a fluorogenic enhancer (Amplify, Amersham, Arlington Heights, IL, USA), and radioactive signals were detected and quantified on a PhosphorImager (Molecular Dynamics, Inc, Sunnyvale, CA, USA). Some experiments were performed in S-buffer (50 mM NaCl, 2 mM $MgCl_2$, 40 mM β-glycerophosphate, 5 mM EGTA, 10 mM HEPES, pH 7.5) instead of HGE + BSA buffer with similar results. PARP was labelled with [^{35}S]methionine (Redivue, Amersham) by *in vitro* transcription and translation with the T3/T7 TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI). Labelled PARP was stored at -70°C with 10 mM DTT to inhibit protein oxidation. PARP cDNA was generated by PCR; the nucleotide sequence was confirmed and subsequently cloned into the Spe I and Bgl II sites of a modified pSVβ plasmid (Clontech, Palo Alto, CA, USA).

Cytosolic calpain-like activity was measured in a fluorometric endpoint assay using 7-amino-4-methylcoumarin (AMC) substrates in 96-well plates.[45,46] Cytosolic extracts (11–33 µg), prepared as described, were incubated with 200 mM LY-AMC or LLVY-AMC in 20 mM Tris/glycerol buffer (Tris, pH 7.5, 5% glycerol, 10 mM DTT, 0.05% BSA) and 0.1 mM or 5 mM $CaCl_2$ in a final volume of 100 µl. Substrate cleavage was analyzed at room temperature at 360 nm excitation/460 nm emission (CytoFluor 2350, Millipore, Bedford, MA) after incubation at 0, 15 and 30 min. In some experiments, purified calpain I (porcine erythrocytes) or purified calpain II (porcine kidney) (Calbiochem, La Jolla, CA) were used in place of the cytosolic extracts. Leupeptin (Sigma) was used to inhibit calpain activity and the cleavage of the AMC substrates by the cytosolic extracts.

Cell viability

Cell viability was assessed with the fluorometric, oxidation-reduction indicator alamarBlue (Alamar Bio-Sciences, Sacramento, CA), which distinguishes between viable cells in oxidized medium and dead cells in reduced medium. Log growth CTLL cells were washed three-times in D-PBS (Life Technologies) and resuspended in cRPMI. The cells were cultured in 96-well plates (Falcon, flat bottom, Becton Dickinson, Lincoln Park, NJ) at $4–8 \times 10^4$ cells per well in 200 µl cRPMI ± rIL-2 [100 U/ml]. At the initiation of culture, the cells were treated with either FMKs, CHX, sodium azide, etoposide or DMSO (FMK vehicle). Unless stated otherwise,

the alamarBlue reagent was incubated with the cells for 9 h before fluorometric analysis. Cell viability (fluorescent intensity) was measured 9–72 h after the initiation of culture.

FMKs and etoposide were diluted to various concentrations in DMSO, and one µl was added per well at the initiation of cell culture. The final DMSO concentration was 0.5% per well. CHX was solubilized in DMSO immediately prior to use, and one µl was added per well at the initiation of culture at various concentrations. Sodium azide was solubilized in dH_2O and used at 0.4% per well, and 20 µl alamarBlue reagent was added per well.

DNA fragmentation and nuclear morphology

Gel electrophoresis was used to determine nucleosomal DNA fragmentation. CTLL cells [1×10^6] were cultured for 18 h with FMKs, DMSO vehicle or CHX in 5 ml cRPMI ± rIL-2 in 10 cm^2 plates. Total cellular DNA was collected from 1×10^6 cells.[47] The total amount of isolated DNA was analyzed by UV light illumination on a 1.8% agarose gel containing 0.5 µg/ml ethidium bromide after electrophoresis at 20 volts for 16 h.

Nuclear morphology was analyzed by fluorescence microscopy. CTLL cells [1×10^6] were cultured with FMKs, DMSO vehicle or CHX in 5 ml cRPMI ± rIL-2 in 10 cm^2 plates. After 18 h, the cells were washed twice in D-PBS, resuspended to 1×10^7 cells per ml in 3% paraformaldehyde and fixed for 20 min on ice. Cells (1×10^6) were air-dried on glass slides, stained with one µg/ml Hoechst 33258 (bisbenzimide, Sigma), mounted in Aqua-poly/mount (Polysciences, Warrington, PA), and analyzed by fluorescence microscopy.

Results

CTLL cells cultured without IL-2 commit to die by apoptosis after 12 h without growth factor

In the absence of IL-2, CTLL cells die by apoptosis, and greater than 95% of the cells are dead after 24 h in growth factor-deprived medium.[40,42] In order to determine the point at which 100% of the growth factor-starved cells can no longer be rescued by the addition of rIL-2, CTLL cells were cultured without rIL-2 and at various times after culture rIL-2 was added to the cells in an attempt to rescue them from death; this time point is defined as the commitment point for death and is the time point where the cell death machinery is irreversibly activated. Cell viability was determined by fluorometry with the viability indicator dye alamarBlue which directly measures metabolic activity. The CTLL cells that were cultured without the rIL-2 growth factor are rescued from death by adding rIL-2 to the cultures within 12 h from the initiation of culture (Figure 1). Approximately 50% of the cells can be rescued when rIL-2 is added to the growth factor-starved cells after about 14–16 h in culture. By 21 h in culture, less than 5% of the cells can be rescued by the addition of rIL-2 to the cultures. The results in Figure 1 directly correlate with trypan blue exclusion results (data not shown).

Cytosolic caspase-3/CPP32-like activity increases in cells undergoing apoptosis

To determine if the caspase family participates in the apoptotic process in CTLL cells, we first evaluated cytosolic extracts from CTLL cells for caspase-3/CPP32-like and caspase-1/ICE-like proteolytic activity. We tested for CPP32 and ICE activities because both of these caspases have been implicated

Figure 1. The commitment point for death in CTLL cells induced by IL-2 starvation is 12 h. CTLL cells [4×10^4 per well] were cultured in IL-2 deficient medium. Between 0 and 21 h following the initiation of culture, the medium was spiked with rIL-2 [100 U/ml]. The metabolic activity indicator dye alamarBlue was added to the cells after 21 h of culture, and the cells were incubated an additional 9 h. Viability (metabolic activity) was determined by fluorometry. Experimental values are reported as means ± SD (*n* = 3).

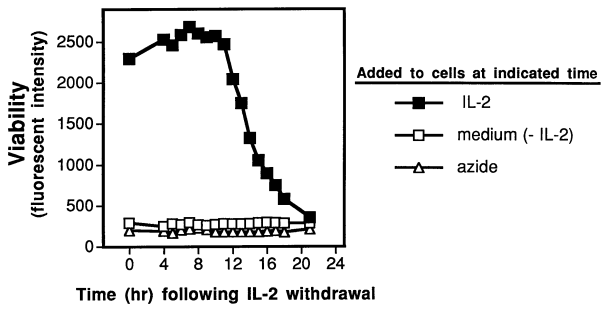
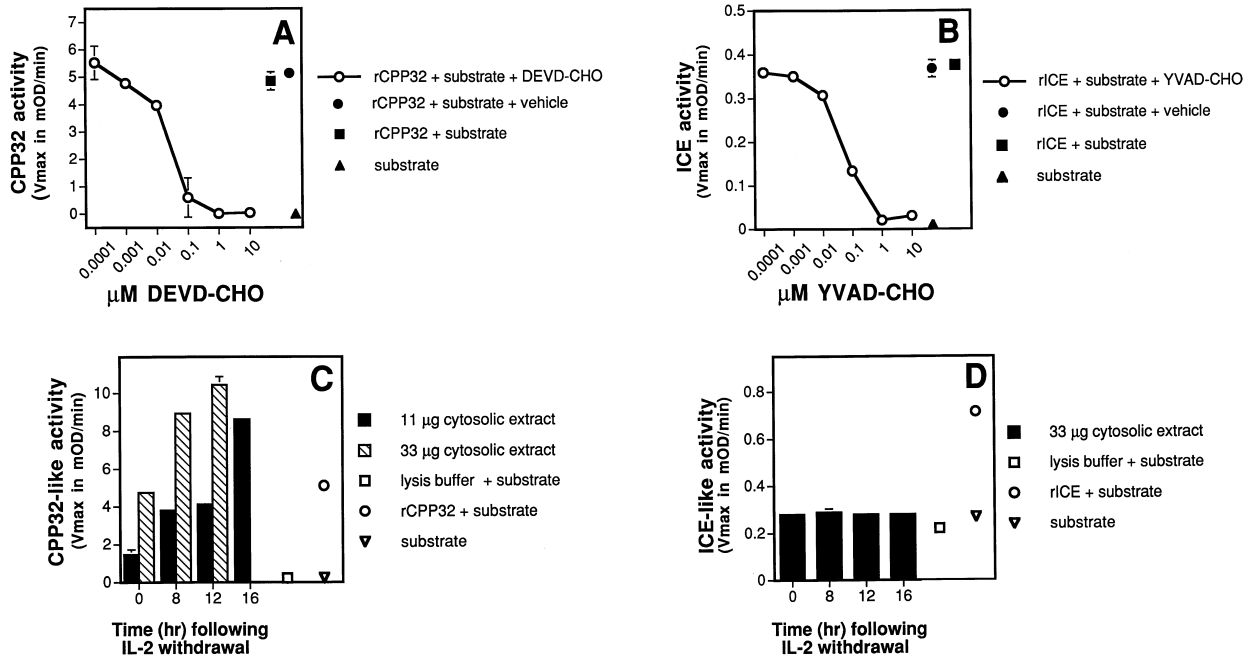
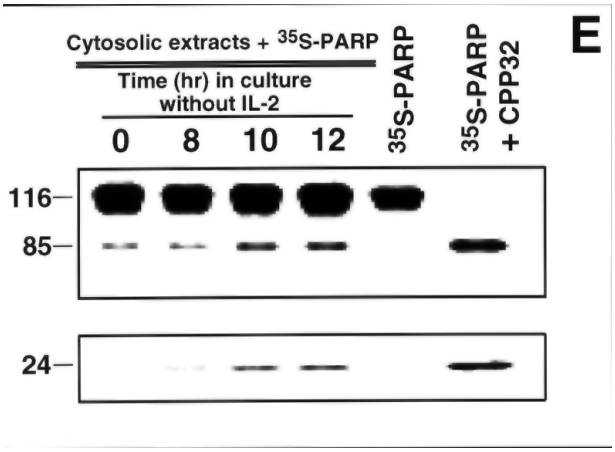


Figure 2. Cytosolic caspase-3/CPP32-like activity increases in CTLL cells undergoing apoptosis following growth factor starvation. (**A**) CPP32 activity and (**B**) ICE activity were measured spectrophotometrically by their abilities to cleave either DEVD-pNA and YVAD-pNA, respectively. Recombinant CPP32 [10 nM] or rICE [50 nM] were incubated in HGE buffer with 0.05% BSA containing either 100 mM DEVD-pNA substrate or 50 mM YVAD-pNA substrate. Some samples were incubated with either the CPP32 inhibitor DEVD-CHO, the ICE inhibitor YVAD-CHO, or DMSO vehicle. Substrate cleavage was analyzed at 30°C at 405 nm (20 min kinetic analysis). (**C**), Cytosolic CPP32-like activity and (**D**) Cytosolic ICE-like activity were measured spectrophotometrically as described above. CTLL cells [2×10^6] were cultured in 5 ml cRPMI without rIL-2 in 10 cm^2 plates. At various times after culture, cytosolic extracts were collected [5×10^7 cell equivalents per ml lysis buffer] and tested for CPP32-like or ICE-like activity. The results for Figures 7A-D are expressed as mean Vmax in mOD/min ± SD ($n = 3$). The cleavage activity of the cell lysis buffer without cell extract is indicated by the group designated *lysis buffer + substrate*. (**E**) Cytosolic CPP32-like activity was measured by the cleavage of ^{35}S-labelled PARP. Cytosolic extracts obtained as described above, were collected from cells cultured for 0, 8, 10 and 12 h without rIL-2. The extracts (5 µg) were incubated with 1 µl of *in vitro* translated ^{35}S-labelled PARP in HGE buffer with 0.05% BSA, or 0.5 µl of ^{35}S-labelled PARP was incubated with 10 nM rCPP32. The reaction mixture was incubated for 10 min at 30°C, diluted with SDS-PAGE buffer, and analyzed on 8% Tris-glycine gels by fluorography.





in apoptosis under physiological conditions in mammalian cells; in addition these assays are well characterized.[16,24,48,49] CPP32 is also highly expressed in cells of lymphocytic origin, rendering it a potential candidate of apoptosis in these cells.[50] CTLL cells were cultured in the absence of rIL-2, and cytosolic extracts were tested at various times after culture for proteolytic activity against peptide substrates. Figures 2A and 2B demonstrate that rCPP32 and rICE cleave DEVD-pNA and YVAD-pNA, respectively. DEVD-CHO and YVAD-CHO, which bind to the active site of CPP32 and ICE, respectively, inhibit the cleavage of the pNA substrates *in vitro*. In CTLL cells, cytosolic CPP32-like activity increased approximately 2.5 to 3-fold from the cells cultured for 8–12 h without rIL-2 as compared to control cells cultured with rIL-2 (0 h time point) (Figure 2C). CPP32-like activity increased about fivefold in extracts from cells cultured for 16 h without rIL-2 as compared to controls. The addition of 0.1 µM DEVD-CHO to

the extracts inhibited the cleavage of DEVD-pNA, whereas the addition of 0.1 μM YVAD-CHO to the extracts had no effect on DEVD-pNA hydrolysis (data not shown). In contrast, ICE-like activity did not change in the cytosol of CTLL cells which were cultured under apoptosis-inducing conditions (Figure 2D), even when 200 μg of cytosolic extract was tested (data not shown). The time points between 8 and 16 h were chosen because the commitment point is about 12 h following culture in IL-2-deficient medium.

CPP32-like activity was also evaluated by cleavage of ^{35}S-labelled PARP, a substrate of CPP32 or a related enzyme during apoptosis.[24,29,32] Cytosolic extracts were obtained from CTLL cells cultured without rIL-2 for 0–12 h. The extracts were incubated with ^{35}S-labelled PARP, and the reaction products were evaluated on SDS-PAGE gels by fluorography (Figure 2E). Full-length ^{35}S-labelled PARP migrates to approximately 116 kDa, and ^{35}S-labelled PARP cleaved by rCPP32 at $DEVD_{216-G217}$ yields two products of approximately 85 and 24 kDa.[24,51,52] A minimal amount of ^{35}S-labelled PARP is cleaved by extracts from cells cultured in rIL-2-containing medium (0 h time point) as compared to the amount of ^{35}S-labelled PARP cleaved from extracts derived from cells cultured for 8, 10 and 12 h without rIL-2. We also tested the extracts for ^{35}S-labelled pro-IL-1β cleavage activity and were unable to detect the generation of any 17 kDa mature IL-1β (data not shown), suggesting that the activity detected in Figure 2D is probably non-specific proteolysis. Overall, the results in Figure 2 indicate that cytosolic extracts from cells cultured between 0 and 12 h without rIL-2 contain proteolytic activity capable of cleaving ^{35}S-labelled PARP to the characteristic 85 and 24 kDa products, and there is a relative increase in ^{35}S-labelled PARP cleaving activity as the cells approach the commitment point of death which is after 12 h in culture without growth factor.

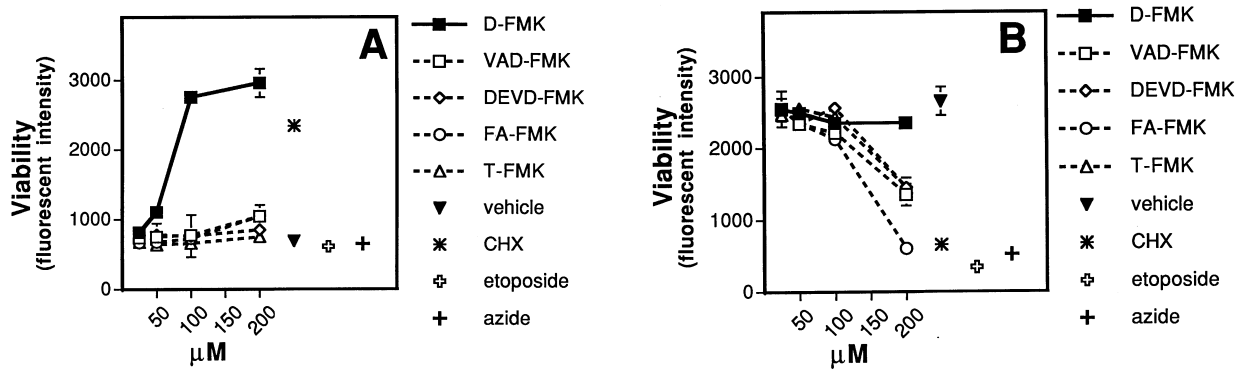
The broad spectrum caspase family inhibitor D-FMK inhibits the death of CTLL cells following IL-2 deprivation; VAD-FMK and DEVD-FMK have no effect

To determine if activated members of the caspase family are involved in growth factor deprivation-induced apoptosis, CTLL cells were cultured in medium without rIL-2 for 24 h. At the initiation of culture, the cells were treated with various inhibitors of the caspase family, which includes DEVD-FMK, VAD-FMK, and D-FMK. We presume DEVD-FMK will inhibit CPP32 activity, since a similar compound DEVD-CHO is reported to be a specific inhibitor of CPP32.[24] VAD-FMK is an inhibitor of both ICE and CPP32.[9,17,53] D-FMK inhibits both ICE and CPP32 *in vitro* with equal potency as well as Fas-mediated apoptosis.[54] Also, similar aspartate-based compounds such as benzyloxycarbonyl-Asp-CH_2OC(O)-2,6,-dichloro benzene and boc-aspartyl (benzyl) chloromethylketone inhibit ICE activity and apoptosis in human myeloid leukaemia U937 cells and CID-9 mammary epithelial cells, respectively.[22,55] Furthermore, the crystal structure of ICE and sequence analysis of the reported caspase family members suggest that D-FMK is a general inhibitor of the caspase family.[56,57] Control FMKs consisted of FA-FMK, which inhibits the cysteine protease cathepsin B,[58] and T-FMK, which serves as a boc-single amino acid-FMK control for D-FMK.

The results in Figure 3A show that D-FMK inhibited the death of CTLL cells in a dose-dependent manner. All other FMKs (VAD-FMK, DEVD-FMK, FA-FMK, T-FMK) did not inhibit death at similar concentrations [25–200 μM]. In addition, the CPP32 inhibitor DEVD-CHO [100–250 μM] did not inhibit the death of CTLL cells (data not shown). Death was also inhibited in the control cells treated with the protein synthesis inhibitor CHX, a potent inhibitor of apoptosis in this system.[40] Cells treated with the DMSO vehicle produced the same amount of fluorescent intensity as cells treated with sodium azide, an inducer of necrosis and etoposide, an inducer of apoptosis. More than 95% of DMSO-, azide- and etoposide-treated cells were dead, whereas 100% of the D-FMK-treated cells were viable as determined by trypan blue staining (data not shown).

To rule out the possibility that the death of CTLL cells was not due directly to FMK toxicity, cells were cultured in rIL-2 containing medium in the presence of the FMKs (Figure 3B). After 24 h, viability was assessed by fluorometry. The viability of cells treated with D-FMK [25–200 μM] was equivalent to that of cells cultured in DMSO vehicle alone. Similar results were obtained with cells

Figure 3. D-FMK inhibits the death of CTLL cells induced by IL-2 starvation. CTLL cells [8×10^4 per well] were cultured (**A**) without rIL-2 or (**B**) with rIL-2 [100 U/ml] for 24 h. At the initiation of culture, the cells were treated with either DMSO vehicle (0.5%), CHX [10 µg/ml], etoposide [50 µM], sodium azide (0.4%), or 25–200 µM fluoromethylketone compounds (D-FMK, VAD-FMK, DEVD-FMK, FA-FMK, T-FMK). Cell viability was assessed by fluorometry using alamarBlue as the fluorogenic indicator. Experimental values are reported as means ± SD ($n = 3$).

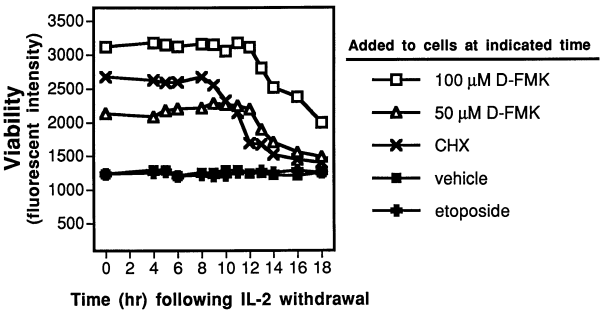


cultured with VAD-FMK, DEVD-FMK, FA-FMK and T-FMK at concentrations below 100 µM. All FMKs were lethal to the cells at concentrations above 100 µM except D-FMK; FA-FMK was the most lethal FMK. Similar to previous results, control cells cultured with both rIL-2 and CHX died after 24 h, and cells cultured with azide or etoposide could not be rescued with rIL-2.[42] In other experiments, the FMK inhibitors were used in combination with each other, and only those cultures that were treated with D-FMK showed enhanced survival. Also, co-treatment of D-FMK-treated cells with either VAD-FMK or DEVD-FMK did not enhance the survival of the cells any more than cells only treated with D-FMK. Overall, the results indicate that D-FMK inhibits growth factor deprivation-induced death of CTLL cells; known inhibitors of CPP32, VAD-FMK and DEVD-FMK, have no effect.

To establish the time that the D-FMK-inhibitable factors are active in the death process, D-FMK was added to IL-2-deprived cells at various times following the initiation of culture. The results in Figure 4 indicate that D-FMK can rescue 100% of the cells from death when added between 0–12 h following the initiation of culture. After 12 h, the cells begin to die which is similar to the results shown in Figure 1 where rIL-2 is used to rescue the cells instead of D-FMK. Interestingly, CHX also delays the death of cells when added after the initiation of culture but only when added within 8 h post-culture. The control cells treated with either vehicle or etoposide are greater than 95% dead as confirmed by trypan blue staining (data not shown). The results in Figure 4 suggest that the mechanism by which D-FMK inhibits apoptosis is not by protein synthesis inhibition like CHX. Moreover, the D-FMK inhibitable executioners of the apoptotic process are activated by approximately 12 h following IL-2 starvation, and important biochemical events associated with apoptosis occur near the 12 h time point.

We also evaluated the length of time in which D-FMK-treated CTLL cells remain viable in

Figure 4. D-FMK rescues CTLL cells from death induced by growth factor starvation when added to the cells by 12 h following the initiation of culture. CTLL cells [8×10^4/well] were cultured without rIL-2. At various times during culture, either D-FMK [50 or 100 µM], CHX [10 µg/ml], DMSO vehicle (0.5%), or etoposide [50 µM] were added to the cells. After 21 h, alamarBlue was added to the cultures which were incubated an additional 20 h. Cell viability was determined by fluorometry. Experimental values are reported as means ± SD ($n = 3$).



medium without rIL-2. The results in Figure 5A indicate that cells cultured with D-FMK in the absence of rIL-2 remain viable up to about 36 h in culture. Between 36–48 h in culture, cell viability begins to decrease with about 50% cell viability after 54 h in culture. Less than 10% of the cells were viable after 78 h in culture. CHX inhibits death of the CTLL cells up to 18 h in culture, but cell viability drops to about 10% between 18–36 h in culture in the CHX treated group. Vehicle-, FA-FMK-, and azide-treated cells do not differ substantially from each other, and they all indicate no protection against growth factor starvation.

D-FMK-treated cells were also cultured in rIL-2 containing medium to determine if D-FMK had an effect on long-term cell viability. CTLL cells treated with either D-FMK or vehicle were equally viable in culture between 18–78 h (Figure 5B). Furthermore, the fluorescent intensity values detected with the D-FMK-treated cells is not due to the compound or the vehicle.

To determine whether readdition of rIL-2 restores growth, D-FMK-treated and vehicle-treated cells were cultured without growth factor for 24 h. The cells were then recultured in medium containing rIL-2 for an additional 24 and 48 h, at which time cell viability was determined. Twenty-four and 48 h after reculture in rIL-2 supplemented medium, the number of viable D-FMK-treated cells increased 1.8-and 5.5-fold , respectively as compared to the starting cell number (Table 1). In contrast, viable cells treated with vehicle were not detected after either 24 or 48 h in culture with growth factor. Control cells treated with D-FMK or vehicle proliferated equally well after 48 h in culture demonstrating that D-FMK is not toxic to dividing CTLL cells. It is difficult to determine the actual number of cells that were rescued by D-FMK treatment or if the rescued cells are physiologically the same as the cells that were continually cultured with rIL-2; however, the results in Table 1 suggest that D-FMK can not only delay, but inhibit, apoptotic death in CTLL cells.

D-FMK inhibits apoptosis characterized by DNA fragmentation and nuclear condensation. The caspase-3/CPP32 inhibitors VAD-FMK and DEVD-FMK have a minimal effect on the apoptotic phenotype

The effect of the FMKs on apoptosis was evaluated by DNA fragmentation and nuclear condensation. CTLL cells were treated with either rIL-2, CHX, FMKs [100 μM], or DMSO vehicle and cultured for 18 h. Genomic DNA was isolated and evaluated by agarose electrophoresis for fragmentation characteristic of apoptotic death in T cells (Figure 6). Cells treated with either vehicle, VAD-FMK, DEVD-FMK, FA-FMK, or T-FMK produced fragmented DNA which gave a characteristic nucleosomal DNA ladder by gel electrophoresis. Cells

Figure 5. D-FMK delays the death of CTLL cells for 36 h following culture without IL-2. CTLL cells [8×10^4 per well] were cultured (**A**) without rIL-2 or (**B**) with rIL-2 [200 U/ml] for 18 to 78 h. At the initiation of culture, the cells were treated with either DMSO vehicle (0.5%), CHX [10 μg/ml], sodium azide (0.4%), D-FMK [100 μM], or FA-FMK [100 μM]. Cell viability was assessed by fluorometry using alamarBlue as the fluorogenic indicator. The cells were incubated with alamarBlue for 6 h before fluorometric analysis, and the values on the *x*-axis indicate the time at which alamarBlue was added to the cells. Experimental values are reported as means ± SD ($n = 3$).

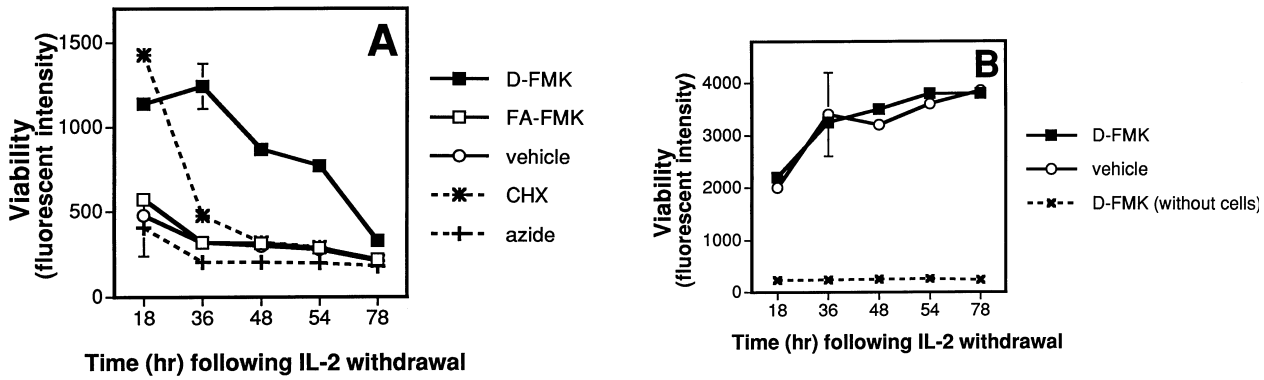


Table 1. D-FMK-treated cells retain their capacity to resume growth upon readdition of growth factor

Treatment[a]	rIL-2	Viable cells after 24 and 48 h in medium following readdition of rIL-2 [200 U/ml][b]	
		24 h	48 h
D-FMK	-	$3.51 \times 10^4 \pm 1.0 \times 10^4$	$1.07 \times 10^5 \pm 8.0 \times 10^4$
Vehicle	-	$< 2 \times 10^4$[c]	$< 2 \times 10^4$
D-FMK	+	NT[d]	$8.35 \times 10^5 \pm 8.0 \times 10^4$
Vehicle	-	NT	$9.81 \times 10^5 \pm 2.2 \times 10^5$

[a] CTLL cells were cultured at 2×10^4 cells per well in one ml in the presence of either D-FMK [100 µM] or DMSO vehicle (0.5%) with (+) or without (-) rIL-2 [200 U/ml]. After 24 h, the cells were washed four-times and recultured in medium containing rIL-2 [200 U/ml]. Trypan blue negative cells were counted after an additional 24 and 48 h in culture.
[b] Viable cells are trypan blue negative. The data are presented as mean ± SD ($n = 3$ wells per group).
[c] Detection limit less than 2×10^4 cells.
[d] NT = not tested.

cultured with CHX produced less detectable DNA fragmentation as compared to the vehicle control group. DNA fragmentation was not detected from cells cultured in rIL-2 containing medium or from D-FMK-treated cells cultured in medium lacking rIL-2. It should be noted that DNA fragmentation evaluated by agarose electrophoresis is qualitative not quantitative.

In order to evaluate the effect of the FMKs on apoptosis in a more quantitative manner, the cells were evaluated by nuclear staining with bisbenzimide (Figure 7A–H). Cells were treated with either the FMKs [100 µM], vehicle, CHX, or rIL-2 and cultured for 18 h before staining with bisbenzimide for fluorescence microscopic evaluation. A majority of cells that were treated with vehicle, T-FMK, FA-FMK, DEVD-FMK or VAD-FMK contained condensed and segmented nuclei characteristic of apoptosis. In contrast, very few cells treated with either D-FMK, CHX or rIL-2 contained nuclei that were condensed or fragmented. The apoptotic nuclei were counted (Figure 7I); 80–90% of the cells treated with vehicle, T-FMK, FA-FMK or DEVD-FMK had apoptotic nuclei. Less than 15% of the nuclei from cells treated with D-FMK, CHX or rIL-2 were apoptotic. Interestingly, 60–70% of the nuclei from VAD-FMK-treated cells showed the apoptotic phenotype: a 10–20% reduction in apoptotic nuclei as compared to the vehicle control. This relatively small inhibition of apoptosis determined morphologically is not reflected by DNA fragmentation on gels (Figure 6) or by the viability (metabolic activity) assays (Figure 3). The discrepancy between nuclear staining with bisbenzimide and the other assays used in this study may be due to different sensitivities of the assays, particularly since nuclear staining involves analysis of individual cells whereas the end points from the other assays are derived from pooled populations of cells. The results in Figures 6 and 7 correlate with the results in Figure

Figure 6. D-FMK inhibits DNA fragmentation associated with apoptosis induced by growth factor starvation. CTLL cells [1×10^6] were cultured in 5 ml cRPMI without rIL-2 in 10 cm^2 plates. At the initiation of culture, the cells were treated with either DMSO vehicle (0.5%), CHX [10 µg/ml], or FMKs (D-FMK, VAD-FMK, DEVD-FMK, FA-FMK, T-FMK) [100 µM]. Control cells were cultured with rIL-2 [100 U/ml] and DMSO vehicle (0.5%). After 18 h, genomic DNA was isolated and nucleosomal DNA laddering was evaluated by agarose gel electrophoresis.

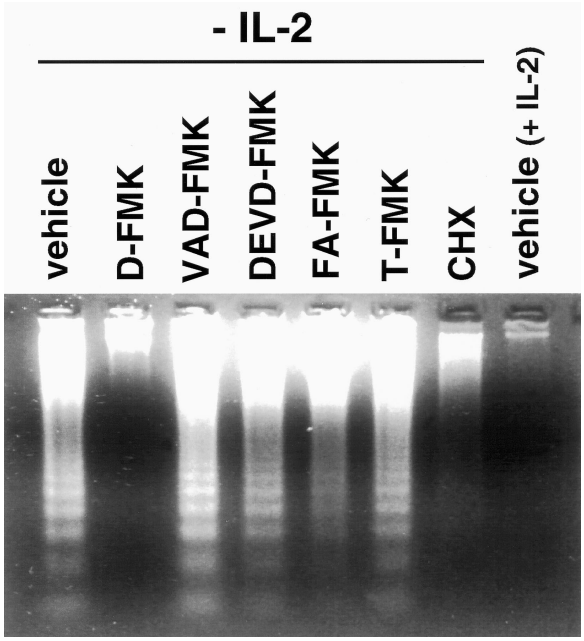
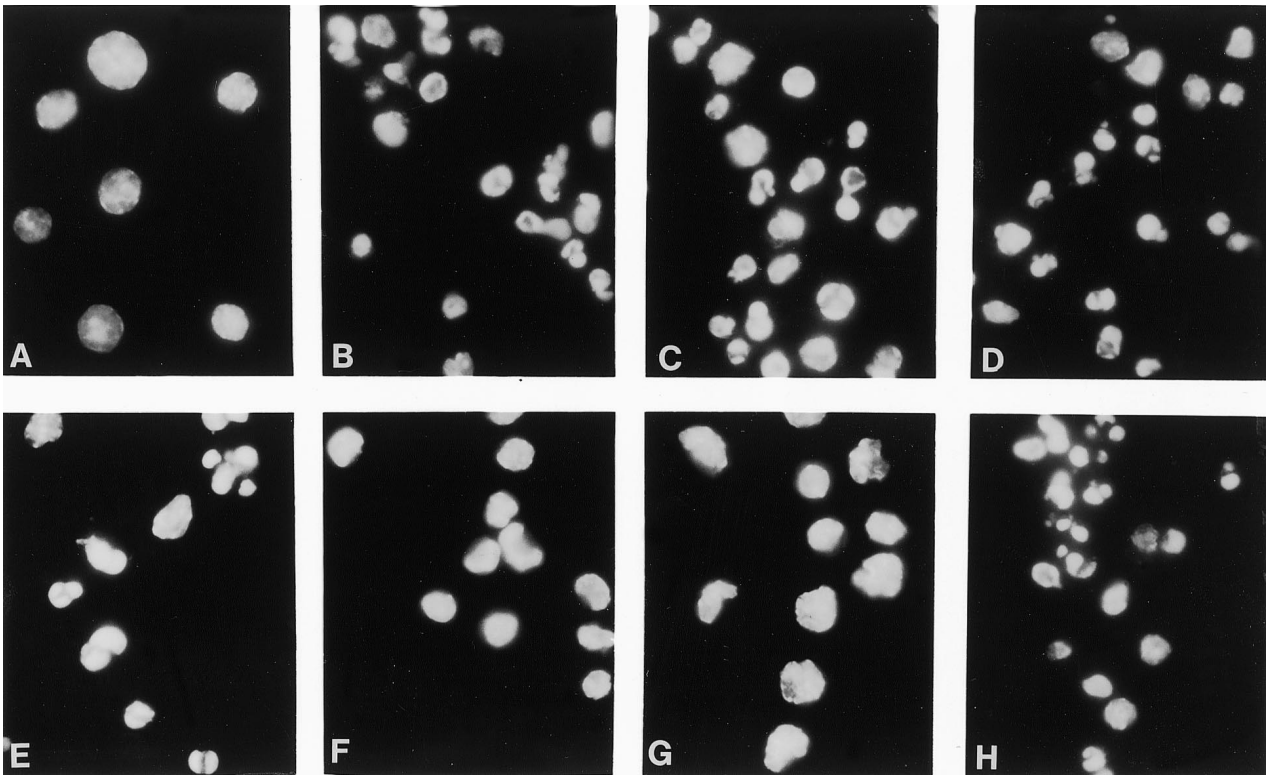
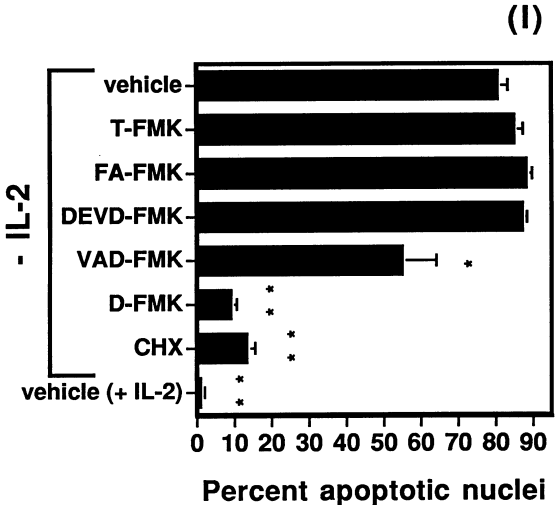


Figure 7. D-FMK inhibits nuclear condensation and segmentation associated with apoptosis induced by growth factor starvation. CTLL cells [1 × 10^6] were cultured in 5 ml cRPMI without rIL-2 in 10 cm^2 plates. At the initiation of culture, the cells were treated with either **A,** DMSO vehicle (0.5%) plus rIL-2 [100 U/ml], **B,** T-FMK [100 µM], **C,** FA-FMK [100 µM], **D,** DEVD-FMK [100 µM], **E,** VAD-FMK [100 µM], **F,** D-FMK [100 µM], **G,** CHX [10 µg/ml], or **H,** DMSO vehicle (0.5%). After 18 h, the cells were stained with bisbenzimide [1 µg/ml] and analyzed by fluorescence microscopy. **I,** The condensed and segmented, bisbenzimide-stained nuclei were counted and expressed as the per cent apoptotic nuclei. The data are reported as mean ± SD (n = 5 microscopic fields).





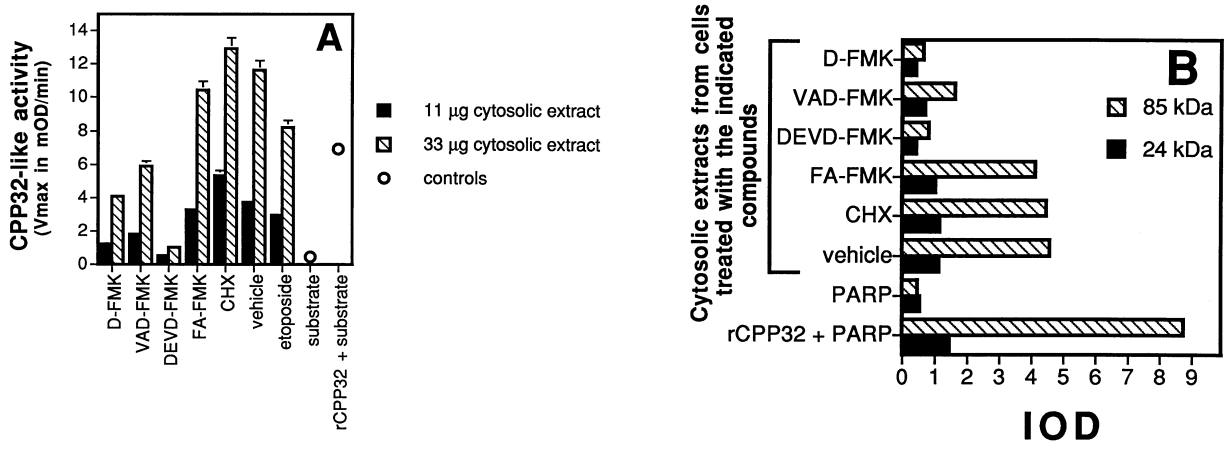
3 and indicate that D-FMK inhibits apoptosis, unlike DEVD-FMK and VAD-FMK which have minimal effects on the apoptotic phenotype.

Caspase-3/CPP32-like activity is inhibited by the apoptosis inhibiting compound D-FMK and also by the compounds which do not inhibit apoptosis: VAD-FMK and DEVD-FMK

If D-FMK inhibits apoptosis in growth factor-deprived CTLL cells by inhibiting homologs of the caspase family, then caspase activity should be reduced in the cytosol of D-FMK-treated cells. CPP32-like activity was tested in the cytosolic extracts from D-FMK-treated cells cultured for 12 h without rIL-2 using the DEVD-pNA and ^{35}S-labelled PARP cleavage assays (Figure 8). The 12 h time point was chosen because apoptosis in CTLL cells can be inhibited by adding either rIL-2 or D-FMK to growth factor starved cells by 12 h after culture (Figures 1 and 4), and CPP32-like activity increases by 12 h under similar culture conditions (Figure 2C).

In the DEVD-pNA cleavage assay, cytosolic ex-

Figure 8. D-FMK, VAD-FMK and DEVD-FMK inhibit cytosolic caspase-3/CPP32-like activity in CTLL cells undergoing apoptosis following growth factor starvation. **A,** Cytosolic CPP32-like activity was measured spectrophotometrically as described in the Figure 2 legend. CTLL cells [2×10^6] were cultured in 5 ml cRPMI without rIL-2 in 10 cm^2 plates. The cells were treated at the initiation of culture with either DMSO vehicle (0.5%), CHX [10 µg/ml], or FMKs (D-FMK, VAD-FMK, DEVD-FMK, FA-FMK) [100 µM]. After 12 h in culture, cytosolic extracts were collected [5×10^7 cell equivalents per ml lysis buffer] and tested for CPP32-like activity by cleavage of DEVD-pNA. The results are expressed as mean Vmax in mOD/min ± SD ($n = 3$). **B,** Cytosolic CPP32-like activity was measured by the cleavage of ^{35}S-labelled PARP. Cytosolic extracts (5 µg), obtained as described, were incubated with 1 µl of *in vitro* translated ^{35}S-labelled PARP in HGE buffer with 0.05% BSA, or 0.5 µl of ^{35}S-labelled PARP was incubated with 10 nM rCPP32. The reaction mixture was incubated for 10 min at 30°C, diluted with SDS-PAGE buffer, and analyzed on 8% Tris-glycine gels by fluorography. *C,* The relative amounts of the 85 kDa and 24 kDa cleavage products detected in Figure 8B were analyzed by optical reflectance. The results are expressed as the integrated optical density (IOD) of the designated band.



tracts from D-FMK-treated or VAD-FMK-treated cells have 2.5- to 3-fold less CPP32-like activity than vehicle-treated or etoposide-treated cells (Figure 8A). The DEVD-FMK-treated cells have approximately 10-fold less activity than the vehicle or the etoposide controls. The FA-FMK-treated group contained slightly less CPP32-like activity than the vehicle control. Interestingly, CHX which inhibits protein synthesis and apoptosis in CTLL cells does not inhibit DEVD-pNA cleavage.

In the ^{35}S-labelled PARP cleavage assay, cleaved ^{35}S-labelled PARP was produced by extracts from cells treated with either vehicle, FA-FMK, or CHX to produce 85 kDa and 24 kDa fragments (Figure 8B). However, cytosolic extracts from cells treated with either D-FMK, VAD-FMK, or DEVD-FMK produced three- to fivefold less cleavage products than the vehicle treated control (Figures 8B, 8C). The DEVD-pNA cleavage results (Figure 8A) correlate with the PARP cleavage results in that D-FMK, VAD-FMK, and DEVD-FMK inhibit cytosolic CPP32-like activity under apoptosis-inducing conditions. However, the level of inhibition is less in the DEVD-pNA assay, probably because the peptide substrate is more likely to be cleaved non-specifically by unrelated proteases than the full-length native PARP protein. In addition, the results in Figure 8 rule out the possibility that VAD-FMK and DEVD-FMK fail to inhibit apoptosis because of poor cell permeability.

The potential for pharmacologic agents to inhibit biochemical processes other than the intended ones always exists, particularly with small, relatively non-specific compounds such as D-FMK. Therefore, we tested the ability of D-FMK to inhibit cytosolic activity of the cysteine protease calpain which has been shown to be involved in some forms of T cell apoptosis.[59] D-FMK did not inhibit calpain in the cytosol of CTLL cells cultured under apoptotic conditions (data not shown). Although the inability of D-FMK to inhibit calpain does not exclude the possibility that D-FMK may inhibit biochemical processes unrelated to the caspase family, it does suggest that D-FMK is not a general inhibitor of cysteine proteases.

Discussion

Although it is not known which specific caspase

members are involved in the apoptotic process under physiological conditions, caspase-3/CPP32 is considered to be a key component because: (i) CPP32 has the highest degree of homology among the caspase members to *ced-3*; (ii) rCPP32 induces nuclear changes characteristic of apoptosis in *in vitro* reconstitution experiments; (iii) rCPP32 cleaves PARP into fragments that are identical to PARP cleavage fragments found in apoptotic cells; and (iv) apoptosis and PARP cleavage are abrogated with inhibitors of pro-CPP32 processing and CPP32 proteolytic activity.[16,24,37,53,60] Our results suggest that apoptosis in CTLL cells does not depend exclusively on CPP32 activity. It is possible that CPP32 activity may participate in the apoptotic process along with other caspase members that are not efficiently inhibited with either VAD-FMK or DEVD-FMK.

Our results correlate with those of Deshmukh *et al.* who demonstrated that D-FMK inhibits apoptosis in sympathetic granule neurons following nerve growth factor withdrawal.[54] Furthermore, the commitment point of rescue with either growth factor or D-FMK is the same indicating that inhibition of apoptosis by growth factor or D-FMK may act by a similar process, which may work mechanistically by inhibiting caspase family protease activity. Indeed, IL-2 enhances *bcl-2* expression in CTLL cells, and *bcl-2* inhibits apoptosis induced by the overexpression of caspase-1/ICE, caspase-2/Ich-1/Nedd2 and caspase-11/Ich-3.[10,20,21,41,61] On the other hand, the commitment point of rescue with CHX is earlier than that of growth factor or D-FMK suggesting that inhibition of protein translation is not the mechanism by which apoptosis is inhibited by D-FMK.

While D-FMK probably inhibits most or all of the caspase family to a certain extent, it is not a general inhibitor of cysteine proteases. Furthermore, a chemically similar inhibitor, Z-D-CH_2DCB, inhibits caspase-1/ICE activity but does not inhibit the cysteine protease cathepsin B.[62] Obviously we can not rule out all potential non-specific effects of D-FMK; however, D-FMK inhibits the cleavage of both DEVD-pNA and ^{35}S-labelled PARP with a similar efficiency as does DEVD-FMK and VAD-FMK. And finally, we can exclude the possibility that all FMKs inhibit caspase family proteases because T-FMK and the cathepsin B inhibitor FA-FMK do not inhibit CPP32-like or ICE-like activity. It is unlikely that the aspartate-specific serine protease granzyme B is required for apoptosis in our experimental paradigm since D-FMK does not inhibit granzyme B,[63] and overexpression of *crmA*, a granzyme B inhibitor,[64] does not inhibit apoptosis in CTLL cells (our unpublished observations).

It is interesting that VAD-FMK does not significantly inhibit apoptosis in CTLL cells following growth factor starvation, despite being an efficient inhibitor of apoptosis in many other mammalian systems.[7,9,16,19,65–67] VAD-FMK inhibits the processing of pro-CPP32,[17] as well as the proteolytic activity of rICE and rCPP32 *in vitro* (our unpublished observations). Based on the latter observation, we presume that VAD-FMK has broader specificity than DEVD-FMK and can inhibit caspases that fall into both ICE-like and CPP32-like categories. Perhaps some members of the caspase family which are involved in CTLL apoptosis do not fall into either category. We hypothesize that D-FMK can potentially inhibit most, if not all, of the caspases to a certain extent, and that its effectiveness in our experimental paradigm is due to the ability of D-FMK to inhibit a member or members of the caspase family which has a significantly different active site than ICE or CPP32, perhaps caspase-2/Ich-1/Nedd2. In fact, inhibition of apoptosis in a growth factor dependent myeloid cell line was achieved with *Nedd2* antisense.[68] Another possibility is that multiple members of the caspase family participate in the apoptotic process in CTLL cells, and efficient inhibition of one or a few caspase members by more specific inhibitors is not sufficient to block apoptosis.

The effectiveness of D-FMK, and ineffectiveness of VAD-FMK and DEVD-FMK, at inhibiting apoptosis in CTLL cells suggests that a caspase family protease is important for apoptosis which has significantly different substrate specificities than ICE-like or CPP32-like proteases. These results are consistent with the finding that T cell apoptosis to various apoptotic stimuli appear normal in CPP32-deficient mice.[69] The results also suggest that CPP32 may not be an appropriate therapeutic target for all pathological conditions involving inappropriate apoptosis. Finally, therapeutic compounds designed to inhibit IL-1β-mediated acute inflammation *via* ICE inhibition may be able to partially inhibit other ICE-like or CPP32-like fam-

ily members without having long-term detrimental effects on apoptosis, *i.e.*, be lyphoproliferative or tumourigenic.

Conclusion

In this report, we examined proteolytic activity and function of the caspase/ICE family during apoptosis using chemical inhibitors with varying specificities. We demonstrate that caspase-3/CPP32-like activity increases in the cytosol of CTLL cells as they undergo apoptosis following IL-2 starvation. It is important to note that we define CPP32-like activity here as DEVD-pNA cleavage, measured spectrophotometrically, and PARP cleavage, measured by fluorography following SDS-PAGE. The increase in cytosolic CPP32-like activity occurs prior to the onset of apoptosis indicating that caspase family proteases are activated during apoptosis in CTLL cells. The pharmacologic inhibitors D-FMK, VAD-FMK and DEVD-FMK, each with varying specificities among the caspase members, inhibit CPP32-like activity from CTLL cells cultured under apoptotic conditions. However, only the broad spectrum caspase family inhibitor D-FMK inhibits apoptosis. The effectiveness of D-FMK, and ineffectiveness of VAD-FMK and DEVD-FMK, at inhibiting apoptosis in CTLL cells suggests that a member of the caspase family is important for apoptosis which has significantly different substrate specificities than ICE-like or CPP32-like proteases. The results also suggest that caspase-3/CPP32 may not be an appropriate therapeutic target for all pathological conditions involving inappropriate apoptosis.

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