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Involvement of Both Caspase-Like Proteases and Serine Proteases in Apoptotic Cell Death Induced by Ricin, Modeccin, Diphtheria Toxin, and *Pseudomonas* Toxin

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We investigated the involvement of caspases and serine proteases in apoptotic cell death induced by ricin, modeccin, diphtheria toxin, and *Pseudomonas* toxin in U937 cells. We found that caspase-3- and caspase-6-like activities, but not caspase-1-like activity, increased during toxin-induced apoptosis. Z-D-CH_2-DCB, a caspase-like inhibitor, completely inhibited the generation of caspase-3- and caspase-6-like activities and blocked all features of apoptosis induced by toxins: nuclear morphological changes, DNA fragmentation, and cytotoxicity. However, three caspase-specific inhibitors, Ac-YVAD-CHO, Ac-DEVD-CHO, and Ac-VEID-CHO, had no effect, even though Ac-DEVD-CHO and Ac-VEID-CHO inhibited the increased caspase-3- and caspase-6-like activity, respectively. These results suggest that the generation of caspase-3- and caspase-6-like activities is redundant, and other caspases distinct from caspase-3 and -6 may be important in toxin-induced apoptosis. Furthermore, serine protease inhibitor, 3,4-dichloroisocoumarine (DCI), abolished the apoptotic cell death and DNA fragmentation caused by toxins, without affecting the increased caspase-3- and caspase-6-like activities. Our results suggest that multiple proteases with different preferences for apoptotic substrates participate in toxin-induced apoptotic death of U937 cells.

Key words: apoptosis, caspase, ricin, serine protease, toxin.

A number of cytotoxic proteins from bacterial (diphtheria, *Pseudomonas*, and Shiga toxin) and plant (ricin, abrin, and modeccin) origins are known to inhibit protein synthesis in eukaryotic cells after entry into the cytosol, resulting in eventual cell death (*1-5*). Diphtheria toxin and *Pseudomonas* toxin inactivate elongation factor-2 by catalyzing the transfer of the ADP-ribose moiety of NAD to the factor; ricin, abrin, modeccin, and Shiga toxin inactivate ribosomes by enzymatically removing a specific adenine residue from 28S RNA of 60S ribosomal subunit. In addition to their ability to inhibit protein synthesis, recent studies have demonstrated that ricin, diphtheria toxin, and *Pseudomonas* toxin induce cell lysis and DNA fragmentation in a process reminiscent of programmed cell death or apoptosis (*6, 7*). Although the mechanism of cell lysis or apoptosis by these toxins is still unclear, it has been reported that ricin-induced apoptotic changes were prevented by cycloheximide and 3-methyladenine, a specific inhibitor of autophagy (*6*). Thus autophagy may be important for ricin-induced cell lysis.

Apoptosis can be induced by a number of unrelated agents, including anticancer agents (*8, 9*), tumor necrosis factor (TNF) (*10*), and Fas (*11*). Cells undergoing apoptosis exhibit characteristic morphological and biochemical changes, including cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation, which result in an oligonucleosomal ladder. Increasing evidence has demonstrated that members of a unique family of cystein proteases play critical roles in controlling apoptotic cell death (*12, 13*). Proteases of this family have distinctive cleavage specificity for aspartic acid in the P1 position and have recently been designated as caspases (cysteinyl aspartate-specific proteases) (*12*). The interleukin-1β-converting enzyme (ICE, *i.e.*, caspase-1) is the first identified member of the caspase family and the mammalian homologue of CED-3, the product of a gene essential for programmed cell death in the nematode *Caenorhabditis elegans* (*14*). Other members of the caspase family including caspase-3 (CPP32/Yama/Apopain) and caspase-6 (Mch2) have been identified. Recent evidence suggests that multiple caspases participate in apoptosis in mammalian cells (*15, 16*), and caspase-3 and caspase-6 have been shown to be the major active caspases responding to different apoptotic stimuli (*17*).

Serine proteases also seem to be implicated in some apoptotic systems. Cytotoxic T lymphocyte-derived serine protease, granzyme B, can activate caspase-3 (*18*). Several nuclear serine-like proteases that could be involved in

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Abbreviations: Z-D-CH_2-DCB, carbobenzoxy-Asp-1-yl-[(2,6-dichlorobenzoyl)oxy]methane; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-YVAD-CHO, acetyl-Tyr-Val-Ala-Asp-aldehyde; Ac-VEID-CHO, actyl-Val-Glu-Ile-Asp-aldehyde; DCI, 3,4-dichloroisocoumarin; MCA, 4-methyl-coumaryl-7-amide; ICE, interleukin-1β-converting enzyme; FBS, fetal bovine serum; PBS, phosphate buffered saline; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

nuclear destabilization during apoptosis have been described (*19, 20*). In our previous studies in MDCK cells, we also found that ricin-induced DNA fragmentation and cell death was inhibited by several serine protease inhibitors (*21*).

To study the involvement of proteases in toxin-induced apoptotic cell death, we examined the effect of various protease inhibitors on cell death and DNA fragmentation induced by ricin and other toxins in human myeloid leukemia U937 cells. Here we report that carbobenzoxy-Asp-1-yl-[(2,6-dichlorobenzoyl)oxy]methane (Z-D-CH_2-DCB), a specific inhibitor of caspase family proteases, and dichloroisocoumarin (DCI), a highly specific serine protease inhibitor, completely blocked apoptosis induced by ricin, modeccin, diphtheria toxin, and *Pseudomonas* toxin, suggesting that both caspases and serine proteases may be involved in a common pathway of apoptotic cell death induced by these protein toxins.

MATERIALS AND METHODS

Materials—Ricin was obtained from Sigma Chemical (St. Luis, MO). Ricin was also isolated from small castor beans as described by Mise *et al.* (*22*). Diphtheria toxin and *Pseudomonas* toxin were purchased from Swiss Serum and Vaccine Institute (Berne, Switzerland). Modeccin was obtained from Inland Laboratories (Austin, TX). The fluorescent tetrapeptide substrates of proteases (Ac-YVAD-MCA, Ac-DEVD-MCA, and Ac-VEID-MCA) and protease inhibitors (Ac-DEVD-CHO, Ac-YVAD-CHO, Ac-VEID-CHO, and Z-D-CH_2-DCB) were obtained from Peptide Institute, Osaka. 3,4-Dichloroisocoumarin (DCI) was obtained from Calbiochem-Novabiochem Intl (La Jolla, CA). [^{3}H]Leucine (60 Ci/mmol) was obtained from NEN Research Products (Boston, MA).

Cell Culture—A human myeloid leukemia U937 cell line was obtained from Riken Cell Bank, Tsukuba. Cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 and 95% air.

Cytotoxicity Assay—Cytotoxicity of toxins was assayed by Alamar blue assay (*23*), which detects dehydrogenases present in living cells in a similar way to MTT assay. In brief, 5×10^4 cells/well in a 96-well plate in RPMI-1640 medium containing 35 μM bovine serum albumin (BSA) were incubated with toxin in the presence or absence of protease inhibitor for 24 h at 37°C, then assayed as described (*23*).

Peptide Cleavage Assay—U937 cells (5×10^6 cell/ml) were incubated with toxin in the presence or absence of protease inhibitor in RPMI-1640 medium containing 35 μM BSA at 37°C for indicated periods of time, then centrifuged, washed twice with PBS, and resuspended in 200 μl of extraction buffer (10 mM HEPES/KOH buffer, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF) as described (*15*). After repeated freezing and thawing, cell debris was removed by centrifugation at 13,000$\times g$ at 4°C for 20 min. The supernatants were incubated at 37°C for 10 min with 10 μM fluorescent substrate, then cleavage of peptide was analyzed with excitation at 380 nm and emission at 460 nm. The specific inhibitor for caspase-1 (Ac-YVAD-CHO), caspase-3 (Ac-DEVD-CHO), or caspase-6 (Ac-VEID-CHO) was added to the reaction mixture at a concentration of 4 μM. Specific caspase-like activities were determined by subtracting the values obtained in the presence of inhibitors.

Measurement of Protein Synthesis Inhibition—Cells in 48-well plates (5×10^5 cells/well) in RPMI-1640 medium containing 35 μM BSA were incubated at 37°C with varying concentrations of ricin in the presence or absence of protease inhibitor. After 3 h of incubation, the medium was replaced by leucine-free medium containing 1 μCi/ml [^{3}H]-leucine by centrifugation, and cells were incubated for 45 min at 37°C. The incorporation of [^{3}H]leucine into perchloric acid/phosphotungstic acid-insoluble materials was determined as described previously (*24*). The results were expressed as percentage of incorporation in control cells incubated without toxin but otherwise treated in the same way.

DNA Fragmentation Assay—Cells (5×10^6 cells/ml) in RPMI-1640 medium containing 35 μM BSA were incubated with indicated concentrations of toxin in the presence or absence of protease inhibitor for 9 h at 37°C. After removal of the medium, cells were washed once with PBS and lysed in 1 ml of lysis buffer (0.2 M NaCl, 10 mM EDTA, 20 mM Tris, 1% sodium dodecyl sulfate, pH 8.0) as described previously (*25*). The lysate was incubated with 250 μg/ml of proteinase K for 10–15 h at 37°C. The solution was then extracted with phenol/chloroform and precipitated with 2.5 volumes of ethanol for 20 min at −83°C, and the precipitate was dried by evaporative centrifugation. The DNA was then dissolved in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 100 μg/ml RNase (DNase free) and incubated for 1 h at 37°C. The DNA of each sample was then analyzed by electrophoresis in 2.0% agarose gel, stained with ethidium bromide, and photographed on a UV illuminator.

Nuclear Staining—Cells (5×10^6 cells/ml) in RPMI-1640 medium containing 35 μM BSA were incubated with toxin for 9 h at 37°C in the presence or absence of protease inhibitor, which was added just before toxin treatment. Cells were washed with PBS, fixed with 1% glutaraldehyde for 30 min at room temperature. The cells were stained with Hoechst 33258 (1 mM) for 5 min and examined by fluorescence microscopy using a microscope (Olympus IMT-2).

RESULTS

Cytotoxicities of Ricin, Modeccin, Diphtheria Toxin, and Pseudomonas Toxin in U937 Cells—In addition to their protein synthesis inhibitory activities, we tested the cytotoxic activities of toxins in U937 cells. After 24 h of incubation with each toxin, cell viability was assessed by Alamar blue assay (*23*). The dose-response curves showed that ricin had a stronger cytotoxicity against U937 cells than other toxins (Fig. 1). The concentrations of toxins required to bring about 100% cell death were: ricin, 10 ng/ml; modeccin, 1,000 ng/ml; diphtheria toxin, 1,000 ng/ml; and *Pseudomonas* toxin, 10,000 ng/ml. These concentrations of toxins were used in later experiments unless otherwise specified.

Caspase-Like Activities in Cytosol from U937 Cells Treated with Ricin and Other Toxins—Recent studies have suggested that caspase family proteases play an important role in many cells undergoing apoptotic death (*12, 13*). To

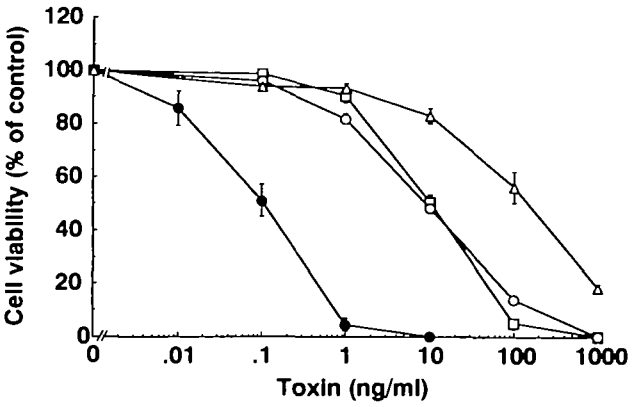

Fig. 1. **Dose-response curves of the cytotoxicities of ricin (●), modeccin (○), diphtheria toxin (□), and *Pseudomonas* toxin (△) in U937 cells.** Cells in 96-well plates (5×10^4 cells/well) were incubated with varying concentrations of each toxin in RPMI-1640 medium containing 35 μM BSA for 24 h at 37°C. Cell viability was assessed by the Alamar blue assay as described under "MATERIALS AND METHODS." Each point is the mean of duplicate determinations.

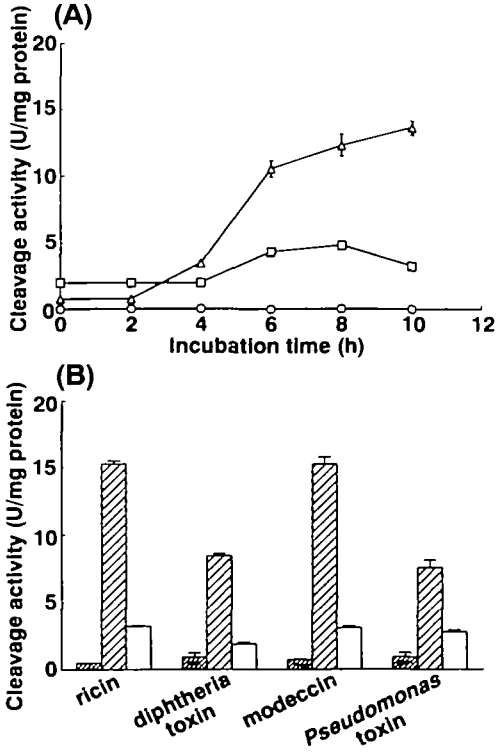

Fig. 2. **Caspase-like activities in cytosol from toxin-treated U937 cells.** (A) Cytosolic extracts were prepared from U937 cells treated at 37°C with 10 ng/ml ricin for the indicated periods of time. Caspase-1- (○), caspase-3- (△), and caspase-6-like (□) activities in the lysates were determined using the fluorescent substrates Ac-YVAD-MCA, Ac-DEVD-MCA, and Ac-VEID-MCA, respectively. One unit corresponds to the activity that cleaves 1 pmol of the respective fluorescent substrate at 37°C in 10 min. (B) U937 cells were treated with ricin (10 ng/ml), modeccin (1,000 ng/ml), diphtheria toxin (1,000 ng/ml), or *Pseudomonas* toxin (10,000 ng/ml) for 9 h. The caspase-1- (▨), caspase-3- (▨), or caspase-6-like (□) activities in the cell extract were assayed.

study the involvement of caspases in ricin-induced apoptosis, cytosolic extracts from U937 cells treated with ricin for various periods were incubated with fluorescent tetrapeptide substrates Ac-YVAD-MCA, Ac-DEVD-MCA, and Ac-VEID-MCA, which are specific substrates for caspase-1, caspase-3, and caspase-6, respectively. As shown in Fig. 2A, the cleavage activity of Ac-DEVD-MCA was gradually increased up to about 10-fold after 9 h of incubation with ricin as compared to the control level in untreated cells, whereas no significant Ac-YVAD-MCA cleavage activity was detected in ricin-treated cells. Control experiments revealed that the Ac-DEVD-MCA cleavage activity in cytosolic extracts from ricin-treated U937 cells was extremely sensitive to Ac-DEVD-CHO and completely insensitive to DCI and Ac-YVAD-CHO at concentration of 100 μM. These results are consistent with the report that cleavage activity of Ac-DEVD-MCA, but not of Ac-YVAD-MCA, was elevated in U937 cells during Fas- and etoposide-induced apoptosis (*26*). A slight but significant increase in Ac-VEID-MCA cleavage activity was also observed during ricin treatment. Examination of the effect of inhibitors revealed that the Ac-VEID-MCA cleavage activity was sensitive to Ac-DEVD-CHO as well as to Ac-VEID-CHO, whereas Ac-DEVD-MCA cleavage activity was insensitive to Ac-VEID-CHO in the same extract, suggesting that the Ac-DEVD-MCA and Ac-VEID-MCA cleavages are mediated, at least in part, by different caspase-like proteases. Regarding the specificity of Ac-DEVD-CHO, it has been reported that VEID-AMC cleavage activity was even

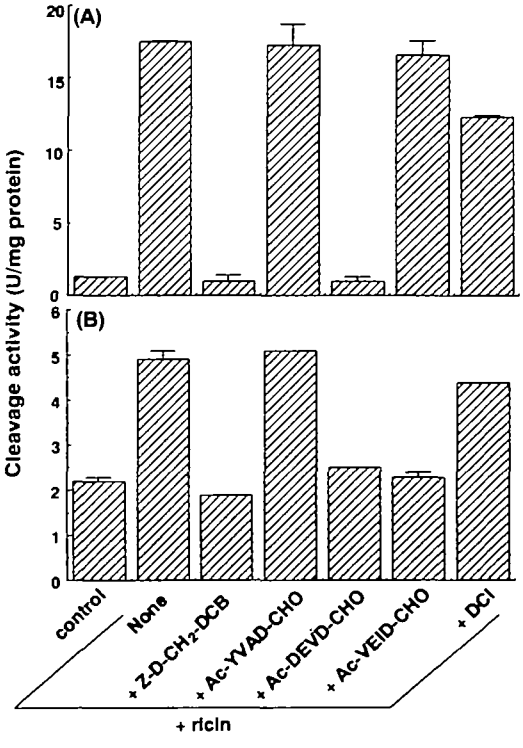

Fig. 3. **Effect of caspase-related protease inhibitors and DCI on the generation of caspase-3- (A) and caspase-6-like (B) activity in ricin-treated U937 cells.** Cells were treated for 9 h with ricin alone or in the presence of 100 μM Z-D-CH_2-DCB, Ac-DEVD-CHO, Ac-YVAD-CHO, Ac-VEID-CHO, or DCI. The caspase-3- or caspase-6-like activity in the cell extract was determined as described in the legend to Fig. 2.

more sensitive to DEVD-fmk than the DEVD-AFC cleavage activity in the extract from HL-60 cells undergoing apoptosis (*27*). Thus, Ac-DEVD-CHO seems to be a effective inhibitor of caspase-6-like activity in addition to caspase-3-like protease. These activation profiles of caspase-like proteases were also observed in cytosolic extracts of U937 cells treated with other toxins. Figure 2B shows the activities of caspase-1-, caspase-3-, and caspase-6-like activities after 9 h of treatment with each toxin.

To test the effect of Z-D-CH_2-DCB (aspartate-based caspase-like inhibitor), Ac-YVAD-CHO (caspase-1 inhibitor), Ac-DEVD-CHO (caspase-3 inhibitor), Ac-VEID-CHO (caspase-6 inhibitor), and DCI (see below) on the caspase-like activities induced in ricin-treated cells, we treated U937 cells with ricin in the presence of 100 μM of each inhibitor and determined the caspase-3- and caspase-6-like activities in the cytosolic extracts. As shown in Fig. 3, Z-D-CH_2-DCB, which would be expected to have a broader specificity for caspases than the tetrapeptide inhibitors, inhibited both caspase-3- and caspase-6-like activities. Ac-DEVD-CHO was also effective in inhibiting both caspases, whereas Ac-VEID-CHO inhibited only caspase-6-like activity. On the other hand, DCI had no significant effect on these activities.

Effect of Caspase-Related Inhibitors and DCI on the Toxin-Induced Apoptotic Cell Death—To further study the involvement of proteases in toxin-induced apoptotic cell death, we examined the effects of caspase-related inhibitors on the cytotoxicities of these toxins in U937 cells. As shown in Fig. 4, Z-D-CH_2-DCB completely prevented cell death induced by ricin and other toxins. However, three caspase-specific tetrapeptide inhibitors (Ac-YVAD-CHO, Ac-DEVD-CHO, and Ac-VEID-CHO) had no effect on toxin-induced apoptotic cell death, even at higher concentration (up to 500 μM) (data not shown). Since Ac-YVAD-CHO and Ac-DEVD-CHO have been shown to prevent Fas-mediated apoptosis (*28*), these results suggest that the apoptotic signal transduction pathway of these protein toxins differs from that of Fas.

Some serine protease inhibitors have been reported to inhibit apoptosis in U937 cells and in other systems (*29-31*). We therefore examined the effect of DCI, a membrane-permeable specific serine protease inhibitor, on the cytotoxicities of toxins in U937 cells in comparison with caspase-related inhibitors. As shown in Fig. 4, DCI strongly inhibited apoptotic cell death induced by these toxins without affecting increased caspase-3 and 6-like activities (Fig. 3).

Effect of Caspase-Related Inhibitors and DCI on the Toxin-Mediated DNA Fragmentation and Nuclear Morphological Changes—Among the most characteristic features of apoptosis are nuclear changes concomitant with

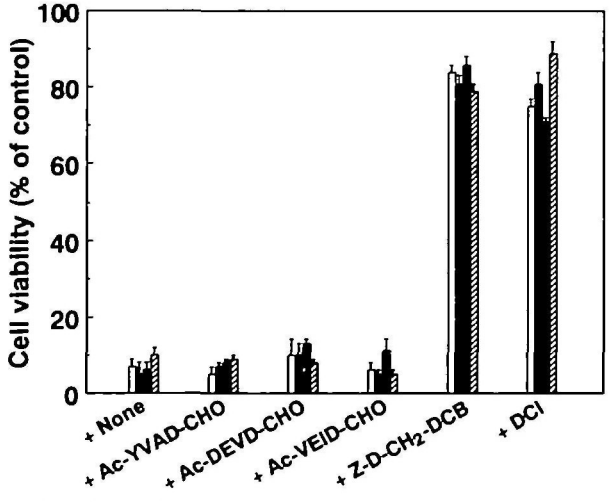

Fig. 4. **Effect of caspase-related protease inhibitors and DCI on the cell death induced by toxins in U937 cells.** Cells were treated for 24 h at 37°C with ricin (10 ng/ml) (▱), modeccin (1,000 ng/ml) (□), diphtheria toxin (1,000 ng/ml) (■), or *Pseudomonas* toxin (10,000 ng/ml) (▨) in the presence or absence of 100 μM Z-D-CH_2-DCB, Ac-DEVD-CHO, Ac-YVAD-CHO, Ac-VEID-CHO, or DCI. Cell viability was assessed by the Alamar blue assay and expressed as a percentage of cell viability of untreated cells.

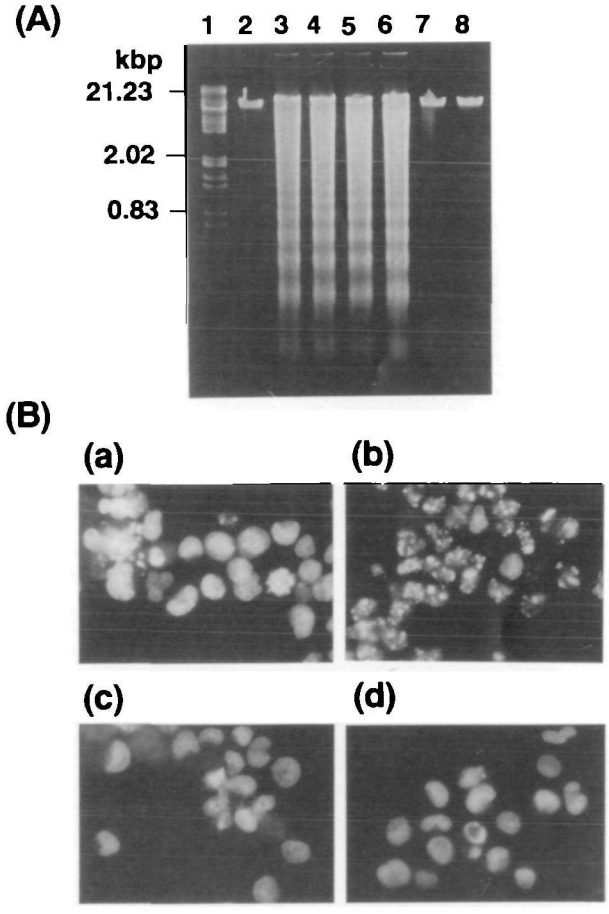

Fig. 5. **Effect of caspase-related protease inhibitors and DCI on the toxin-induced DNA fragmentation and nuclear morphological changes in U937 cells.** (A) Cells were treated for 9 h at 37°C with ricin alone or in the presence of 100 μM Z-D-CH_2-DCB, Ac-DEVD-CHO, Ac-YVAD-CHO, Ac-VEID-CHO, or DCI. DNA was extracted and analyzed on 2.0% agarose gel as described under "MATERIALS AND METHODS." Lane 1, molecular weight standards (kbp); Lane 2, control untreated cells; Lanes 3-8, cells treated with ricin (10 ng/ml) alone (lane 3) or in the presence of 100 μM Ac-YVAD-CHO (lane 4), Ac-DEVD-CHO (lane 5), Ac-VEID-CHO (lane 6), Z-D-CH_2-DCB (lane 7), or DCI (lane 8). (B) (a) Control (untreated cells). Cells were treated for 9 h at 37°C with ricin (10 ng/ml) alone (b) or in the presence of 100 μM Z-D-CH_2-DCB (c) or DCI (d). After treatment, cells were fixed and stained with 10 μg/ml of Hoechst 33258, and observed under a fluorescence microscope.

TABLE I. **Effect of Z-D-CH_2-DCB and DCI on cellular protein synthesis and on the protein synthesis-inhibitory activity of ricin.**

Treatment	Protein synthesis (% of control)
None	100.0
+Z-D-CH_2-DCB (100 μM)	83.7±0.1
+DCI (100 μM)	4.9±1.9
+Ricin (10 ng/ml)	2.6±0.2
+Z-D-CH_2-DCB (100 μM), +Ricin (10 ng/ml)	3.6±1.2
+DCI (100 μM), +Ricin (10 ng/ml)	2.3±1.2

Cells in 48-well plates (5×10^5 cells/well) were incubated at 37°C with varying concentrations of ricin in the presence or absence of protease inhibitor. After 3 h of incubation, the medium was replaced by leucine-free medium containing 1 μCi/ml [^{3}H]leucine, and cells were incubated for 45 min at 37°C. The results were expressed as percentage of incorporation in control cells incubated without toxin but otherwise treated in the same way.

DNA degradation and the formation of a DNA ladder due to internucleosomal cleavage of chromosomal DNA. Analysis by agarose gel electrophoresis of DNA extracted from U937 cells treated with ricin revealed that ricin induced the degradation of DNA and the formation of a ladder as previously reported (*21*). As shown in Fig. 5A and B, Z-D-CH_2-DCB and DCI suppressed ricin-induced DNA fragmentation as well as apoptotic nuclear morphological changes as examined by Hoechst 33258 staining. However, none of the three tetrapeptide caspase inhibitors blocked ricin-induced DNA fragmentation (Fig. 5A). DNA fragmentation induced by other toxins was also prevented by Z-D-CH_2-DCB and DCI (data not shown).

Effect of Z-D-CH_2-DCB and DCI on Cellular Protein Synthesis—We examined the effects of Z-D-CH_2-DCB and DCI on cellular protein synthesis and on the protein synthesis inhibitory activity of ricin in U937 cells. Although no significant effect of Z-D-CH_2-DCB on protein synthesis and ricin activity was observed, DCI itself inhibited cellular protein synthesis (Table I), and the IC_{50} was estimated to be 9.8 μM from the dose-response curve (data not shown). At present, we do not know the mechanism by which DCI inhibits protein synthesis. However, we cannot rule out the possibility that this activity of DCI is partly responsible for its protective effect against ricin-induced apoptosis, since cycloheximide has been shown to inhibit ricin-induced apoptosis (*6, 32*).

DISCUSSION

Recent studies have demonstrated that ricin and other toxins such as diphtheria toxin and *Pseudomonas* toxin trigger a sequence of events that ultimately causes apoptosis, even though these toxins have different biochemical mechanisms of protein synthesis inhibition (*6, 7, 21, 25*).

Apoptosis is a specific form of cell death that is generally observed under normal physiological conditions in response to various stimuli. Apoptosis is also observed when tumor cells are exposed to antitumor agents (*8, 9*). It is generally considered that apoptosis is an active process and may involve the activation of an endogenous Ca^{2+}/Mg^{2+}-dependent endonuclease that degrades nuclear DNA into discrete oligonucleosome-sized units (DNA ladder formation) (*33-35*). Although the mechanism by which the endonuclease is activated during apoptosis remains poorly defined, several lines of evidence have suggested that proteolysis plays an important role, and it appears that an essential component is caspases, a family of cysteine proteases (*36, 37*). Ten caspases have been identified in humans so far (*38*). A growing body of evidence suggests that multiple caspases are involved in the same apoptotic cell. For instance, several caspases were found to be processed in apoptotic cells (*36*), suggesting their activation is linked to apoptosis. Furthermore, studies in a cell-free system provided evidence that apoptotic nuclear changes require more than one caspase (*39*).

In this study we demonstrated that Z-D-CH_2-DCB completely inhibited the apoptotic cell death and DNA fragmentation in U937 cells mediated by ricin and other protein toxins (Figs. 4 and 5). Consistent with our results, Z-D-CH_2-DCB has been shown to block apoptotic cell death induced by many different stimuli, such as etoposide, camptothecin, 1-β-D-arabinofuranosyl-cytosine, adriamycin, TNF-α, anti-Fas antibody, and staurosporine (*29*). Thus, these findings together with our results suggest that Z-D-CH_2-DCB-inhibitable proteases are key mediators of the common signaling pathway in apoptosis in mammalian cells responding to a variety of different cytotoxic stimuli including protein toxins. At present, we cannot identify the cellular target of Z-D-CH_2-DCB. Since caspases have the common feature of cleaving residues next to Asp, one possibility is that the aspartate-based protease inhibitor Z-D-CH_2-DCB effectively inhibits all caspase-like proteases, as it inhibited caspase-3- and caspase-6-like activities in ricin-treated cells (Fig. 3). Another possibility is that Z-D-CH_2-DCB blocks a novel apoptogenic protease which may not be among the known caspase family proteases.

In contrast, three caspase-specific tetrapeptide inhibitors, Ac-YVAD-CHO, Ac-DEVD-CHO, and Ac-VEID-CHO, had no effect on toxin-induced apoptosis, even at higher concentration (500 μM) (data not shown). The possibility that inability of these tetrapeptide inhibitors, especially Ac-DEVD-CHO and Ac-VEID-CHO, to prevent the toxin-mediated apoptosis is due to their poor membrane permeability is unlikely, since Ac-DEVD-CHO and Ac-VEID-CHO added extracellularly at 100 μM inhibited respectively the caspase-3- and caspase-6-like activities in ricin-treated cells (Fig. 3). Furthermore, it has been shown that Ac-YVAD-CHO and Ac-DEVD-CHO at 300 μM almost completely suppressed Fas-mediated apoptosis when used on whole cells (*28*). In addition, 120 μM Ac-DEVD-CHO has been reported to inhibit apoptosis in intact Jurkat cells, whereas Ac-YVAD-CHO was without effect (*40*). Therefore, our results suggest that activation of caspase-3- and caspase-6-like activities may not be directly responsible for the toxin-mediated apoptosis. The speculation that caspase-3 has a redundant effector function is supported by the recent report that the ability of many cell types to undergo apoptosis is not affected in caspase-3-deficient mice (*41*). This redundancy was consistent with the finding that caspases other than caspase-3 can cleave poly(ADP-ribose) polymerase (PARP), an apoptotic substrate of caspase-3 (*42*). Although there is little doubt that caspases are a critical element of the apoptotic machinery in many systems, which caspases are involved is probably specific to cell types and apoptotic stimuli. Regarding the protease activation cascade, there is a possibility that the

apoptotic signalling pathway of ricin and other protein toxins is somehow distinct from other systems in which caspase-1, -3, and -6 are profoundly involved. Recently, apoptosis-inducing factor (AIF) has been partially characterized as a 50-kDa protease released from mitochondria, whose activity on isolated nuclei is inhibited by *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk), an inhibitor of ICE-like proteases, but not by specific inhibitors of ICE (Ac-YVAD-CHO) and CPP32/Yama (Ac-DEVD-CHO) (*43*). Like Z-D-CH_2-DCB, Z-VAD.fmk is thought to be a less specific protease inhibitor than conventional tetrapeptide inhibitors, and it is also an efficient inhibitor of apoptosis in mammalian cells (*44*). Therefore, one can speculate that an analogue of AIF is also involved in ricin-induced apoptosis, and that such an AIF may be a Z-D-CH_2-DCB-inhibitable protease. Further studies are required to clarify this point.

It has been reported that serine proteases are also implicated in some different apoptotic systems. In accordance with our previous report that serine protease inhibitors prevented the toxin-mediated apoptotic death of MDCK cells (*21*), DCI inhibited the apoptotic cell death and DNA fragmentation induced by toxins and suppressed ricin-induced nuclear morphological changes in U937 cells (Fig. 5). Since DCI did not affect the elevation of caspase-3- and caspase-6-like activities, its intracellular target may be distinct from those of caspase-related proteases. Furthermore, DCI has been shown to inhibit DNA fragmentation in etoposide-treated U937 cells without preventing caspase-3 activation and PARP cleavage (*31*). Based on these findings and our present results, it can be speculated that DCI-inhibitable proteases may act at a site downstream of caspase-3-like activity, or else on a separate parallel signal transduction pathway that is independent of caspase-3. In this regard, it has been reported that TPCK could also inhibit TNF- or UV light-induced apoptosis in U937 cells without preventing activation of caspase-3-like activity (*45*). According to the literature, the target of TPCK is 24-kDa serine protease (AP24), which is activated during apoptosis and has the capacity to activate internucleosomal DNA fragmentation in isolated nuclei. Unlike caspase family proteases such as caspase-1 (*46*) and caspase-3 (*15*), AP24 does not require the presence of cytosolic components to activate nuclear DNA fragmentation in cell-free systems. Whether this protease is also DCI-sensitive has not been examined, but such a serine protease, which is probably not related to the caspase family, may play a key role in transmitting apoptotic signals from the cytosol to the nucleus, where it directly or indirectly activates endogenous endonucleases resulting in DNA degradation.

In addition to its general function as a serine protease inhibitor, DCI was found to inhibit cellular protein synthesis (Table I). Since cycloheximide has been shown to inhibit ricin-induced apoptosis (*6, 32*), it is possible that protein synthesis inhibition by DCI is partly responsible for its preventive effect on ricin-induced apoptosis.

In conclusion, our results suggest that multiple proteases with different substrate specificity are involved in the common pathway leading to apoptotic cell death induced by ricin, modeccin, diphtheria toxin, and *Pseudomonas* toxin.

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