Protease Involvement in Fodrin Cleavage and Phosphatidylserine Exposure in Apoptosis*

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A detailed kinetic analysis of three extranuclear end points of apoptosis, phosphatidylserine exposure, α-fodrin degradation, and plasma membrane blebbing, was performed and compared with nuclear fragmentation and the activation of the interleukin-1β-converting enzyme (ICE)-like proteases in Jurkat T lymphocytes stimulated by anti-Fas monoclonal antibody (anti-Fas mAb) and in monocytic U937 cells stimulated by tumor necrosis factor (TNF) and cycloheximide. Phosphatidylserine exposure was quantitated by plasma clotting time, as well as annexin V-fluorescein isothiocyanate binding, and the ICE-like protease activity was examined by the cleavage of a specific fluorogenic peptide substrate Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin. VAD-chloromethylketone (VAD-cmk), an inhibitor of ICE-like proteases, effectively inhibited ICE-like activity in both cell types studied, whereas the calpain inhibitor calpeptin was ineffective. VAD-cmk also effectively inhibited all three extranuclear events, as well as nuclear fragmentation, in Jurkat cells stimulated by anti-Fas monoclonal antibody, indicating that ICE-like proteases play an important role in the regulation of this apoptotic system. Calpain inhibitors were ineffective in this system. TNF-induced extranuclear and nuclear changes in U937 cells were inhibited by calpeptin but were not as effectively inhibited by VAD-cmk as in Jurkat cells. This suggests that ICE-like enzymes predominate in anti-Fas monoclonal antibody-stimulated Jurkat cells, whereas proteases affected by calpain inhibitors as well as the ICE-like enzymes are involved in the signaling of apoptotic events in TNF-induced U937 cells. Importantly, the two apoptotic systems seem to be regulated by different proteases.

The apoptotic process, beginning with receptor-mediated signaling, leading to cytoplasmic and nuclear changes and culminating in the phagocytosis of dying cells, defines an important biological continuum. Although morphological changes have been well characterized, it is necessary to investigate how extranuclear and nuclear events relate to each other temporally and how they relate to the earlier signaling events. Characteristic morphological changes include plasma membrane blebbing, chromatin condensation and fragmentation into high molecular weight (HMW)1 (50–300-kilobase pair) and oligonucleosomal-length (180-base pair) DNA fragments, and the formation of apoptotic bodies (1, 2). PS exposure on the cell surface is another very important change occurring in cells during apoptosis. It has been identified as a trigger for stimulating the phagocytosis of apoptotic cells by macrophages, thus preventing secondary necrosis and inflammation of the surrounding tissue (3, 4). PS and phosphatidylethanolamine are actively confined to the inner cytoskeletal leaflet of the plasma membrane by aminophospholipid translocase. Inhibition of this enzyme leads to disruption of membrane phospholipid asymmetry and PS exposure (5–12). Studies in platelets, in erythrocytes, and more recently in apoptotic lymphocytes suggest that a second enzyme is required for transbilayer lipid scrambling (12–17); however, further studies in apoptotic cells are required to understand the mechanism triggering this important apoptotic end point.

The signal transduction mechanisms involved in the receptor-mediated initiation of apoptosis are still not well defined. Proteolytic activity plays an important role in apoptosis, and proteases from the ICE-like family appear to be essential for the execution of the cell death process (18–20), since overexpression of any one of these proteases induces apoptosis in cultured mammalian cells (21). Studies with enucleated cells and reconstituted in vitro systems have emphasized the importance of cytoplasmic regulatory mechanisms (22–25). It has been shown that the ICE-like proenzymes (Ich-1/Nedd-2, Mch2, prICE/CPP32/apopain/YAMA, and Mch3/ICE-LAP3/CMH-1) (23, 26–36) are important regulators of cytoplasmic processes, capable of specifically cleaving both relevant cellular substrates and zymogens in cellular models of receptor-mediated apoptosis. The consequence of proenzyme activation, which may result in either the sequential or parallel cascades of proteolytic activity, is currently an area of intense study. Activation of cytosolic enzymes, FLICE/MACH, Mch2α, CPP32/apopain, and Mch3α, are clearly early events in the initiation of the apoptotic signaling pathway (37, 38). However, it is still not known whether one of these enzymes is the penultimate protease at the apex of a proteolytic cascade, ultimately responsible for changes in cell homeostasis leading to apoptosis.

Calpain, a cytoplasmic, calcium-dependent neutral protease, activated by micromolar levels of intracellular Ca2+, has been reported to be involved in several apoptotic models (39–41), although the exact role of calpain in apoptosis remains obscure.

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1 The abbreviations used are: HMW, high molecular weight; mAb, monoclonal antibody; ICE, interleukin 1β-converting enzyme; TNF, tumor necrosis factor; PS, phosphatidylserine; PBS, phosphate-buffered saline; RVV, Russell’s viper venom; VAD-cmk, VAD-chloromethylketone; DEVID-AMC, Ac-Asp-Glu-Val-Asp-COOH, ICGmax concentration of inhibitor to give 50% of maximum response; FITC, fluorescein isothiocyanate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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Fodrin (nonerythroid spectrin), one of the proteins cleaved during apoptosis (42), is a cytoskeletal protein found in virtually all eukaryotic cell types (43) and is thought to be responsible for coupling a variety of membrane-spanning cell surface proteins to cytoplasmic elements (44). Calpain has been reported to cleave fodrin into fragments similar to those generated during apoptosis, and this is thought to result in a reduced ability to cross-link actin filaments (45–47). Plasma membrane blebbing and cellular fragmentation have been shown to depend upon actin polymerization (48) and could thus be dependent on fodrin integrity. It has been suggested that intact fodrin could also be associated with the maintenance of membrane lipid asymmetry (49–52) by anchoring PS at the cytofacial membrane, but contrary evidence has also been published (53–56). Both ICE-like proteases and calpain have restricted substrate cleavage specificity, and hence it is thought that they may play a role in regulating cellular events rather than mediating general protein breakdown (40, 57, 58). It is not known whether receptor-mediated calpain activity occurs before or after the activation of other proteases such as the ICE family.

In the present study we compare the kinetics of nuclear chromatin fragmentation with three extranuclear apoptotic events, PS exposure, fodrin proteolysis, and plasma membrane blebbing, in relation to the activation of the ICE-like proteases in two apoptotic cell models. Since it is likely that these events are controlled by cytoplasmic regulatory mechanisms, we have investigated the contribution of calpain-dependent as well as ICE-like enzyme-dependent pathways using specific inhibitors. Calpain inhibitors were shown to be effective in blocking apoptosis in monocytic U937 cells stimulated by TNF, while inhibitors of the ICE-like proteases inhibited apoptosis in Jurkat T lymphocytes stimulated by anti-Fas mAb, indicating that different proteases may be involved in certain cell types.

EXPERIMENTAL PROCEDURES

Materials—Anti-human Fas monoclonal antibody (IgM CH-11 clone) was purchased from Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan). Anti α-fodrin monoclonal antibody was from Affiniti. TNF (human) was a kind gift from Dr. Grace Wong, Genentech Inc. (San Francisco, CA). Calpain inhibitors I and II were obtained from Boehringer Mannheim. Calpeptin was from Bioel. VAD-cmk was purchased from Enzyme Systems Progress. BOC-aspartyl (benzyl) chloromethylketone was a kind gift from Dr. Roy A. Black (Immunex Corp.). Annexin V-FITC was from Nenxis. Propidium iodide was from Sigma. All other reagents were of the highest purity available.

Cell Culture—Jurkat cells (human leukemic T cell line) and U937 cells (human myeloid leukemia cell line, from the Microbiology and Tumor Biology Center, Karolinska Institute) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO2 in air at 37°C. The cells were maintained in a logarithmic growth phase by routine passage every 2–3 days.

Preincubation with Protease Inhibitors—Cells were preincubated with protease inhibitors for 60 min before the addition of apoptotic stimuli. In a previous study with radiolabeled calpain inhibitor I, it was determined that a significant amount of inhibitor was taken up into the cells within 120 min (59). A 60-min preincubation time was chosen, since an effective inhibitory response was achieved in this time. The various protease inhibitors did not adversely affect cell viability over the time courses studied. After 6 h, calpeptin (50–100 μg/ml) alone induced some apoptosis, as did calpain inhibitor I alone after 7–8 h.

Assessment of Apoptotic Nuclei—The cell suspension (3 × 106 cells) was spun at 3000 × g (5 min), and the pellet was resuspended in paraformaldehyde (4% in PBS) and spread on a poly-L-lysine (100 μg/ml) treated glass microscope slide and left to dry. The cells were rehydrated in H2O, dried, and stained with Hoechst 33342 (10 μg/ml) in the dark for 10 min. The slides were then washed in PBS, dried in the dark, and covered with glycerol:PBS (50:50, v/v) and a coverslip. The fluorescence nuclei were viewed with a Leitz Diaplan fluorescence microscope (Wetzlar, Germany), and the nuclei present in a field of at least 100 cells were analyzed.

Determination of DEVD-AMC Cleavage—The ICE-like enzyme activity was investigated by the use of the substrate DEVD-AMC. AMC cleavage was measured fluorometrically by a modification of the method described by Nicholson (60). Cell lysate and substrate were combined in a standard ICE reaction buffer (100 mM HEPES, 10% sucrose, 5 mM diithiothreitol, 0.1% CHAPS, pH 7.25) (61). The specific cleavage of the fluorogenic peptide substrate DEVD-AMC was monitored by AMC liberation using 355-nm excitation and 460-nm emission wavelengths. Fluorescence units were converted to pmol of AMC using a standard curve with increasing doses of AMC against fluorescence (61).

Preparation of PS Liposomes—Phosphatidylcholine liposomes containing 0–30 mol% of PS were prepared by sonication. The appropriate amounts of PC and PS stock solutions in methanol:chloroform were transferred to a glass tube, and the solvent was evaporated under a stream of N2. Lipids were then dissolved in PBS and sonicated on ice bath with a Soniprep 150 MPE probe sonifier until the solution was clear (~8 min). The solution was then spun at 18,000 × g to remove titanium particles. The total concentration of phospholipids (PS and PC) in the liposomes was 160 μM.

Measurement of PS Exposure—PS exposure was measured as an increase in plasma clotting time using the Russell’s viper venom (RVV) assay (4, 6). The time to clot formation was measured with a coagulometer (Amelung, Schnitger and Gross). All measurements were done in triplicate. Before each assay of PS exposure, cell viability was measured using trypan blue exclusion, and the cells were determined to be >95% viable. PS exposure was assayed by RVV clotting time only before significant cellular fragmentation had occurred, when the number of viable cells was constant, such as decreasing cell count (increasing cell fragmentation) an artificial increase in clotting time (decrease in PS exposure) was evident.

PS exposure was also measured directly by the binding of annexin V-FITC using the ApoTest binding kit, containing annexin V-FITC and binding buffer. Cells were also stained with propidium iodide (100 μg/ml). The cells were analyzed with a Becton Dickinson FACScan flow cytometer equipped with a 15-mW, 488-nm air-cooled argon laser. Dot plots were constructed in order to determine the percentage of apoptotic cells showing annexin V-FITC binding and those permeable to propidium iodide. Histograms of the change in mean fluorescence intensity of annexin V-FITC in control and apoptotic cells are shown under “Results.”

Assessment of Plasma Membrane Blebbing—The percentage of blebbing cells was determined using light microscopy by counting in a field of at least 100 cells. Blebbing was defined by the appearance of distinct protrusions of the plasma membrane. Blebbing was followed by cellular fragmentation, and it was not possible to accurately count blebbing once this fragmentation had begun.

Preparation of Cell Lysates—Cells were washed with ice-cold PBS, pH 7.1, containing 100 μM phenylmethylsulfonyl fluoride and pelleted at 200 × g. Cell pellets were resuspended in lysis buffer (62.5 mM Tris-HCL, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 100 μM phenylmethylsulfonyl fluoride) and boiled in a water bath for 5 min. Lysates were stored at −20 °C until further analyzed.

Electrophoresis and Western Blotting of Proteins—Proteins were separated under reducing conditions for 2 h at 110 V in 6% SDS-polyacrylamide gels and then Western blotted at 100 V for 2 h. Blots were blocked overnight in high salt buffer (50 mM Tris base, 500 mM NaCl, 0.05% Tween 20) containing 3% bovine serum albumin and then incubated for 1 h with fodrin antibody (against the fodrin α subunit) diluted in high salt buffer. After washing the blots 4 times for 5 min in high salt buffer, they were incubated with a peroxidase-conjugated secondary antibody (Pierce), and bound antibody was detected by enhanced chemiluminescence (Amersham Corp.). The intensity of luminescence recorded on the ECL film was then measured with a Shimadzu CS-9001PC scanner.

Analysis of HMW DNA Fragments—After appropriate incubations, 106 cells were centrifuged at 2000 × g for 5 min, and pellets were resuspended in 50 μl of PBS and mixed with 50 μl of low melting point agarose (1%). The agarose plugs were digested for 48 h with Pronase (1 mg/ml) at 50°C. The HMW DNA fragments were analyzed using field inversion gel electrophoresis as described previously (63).

RESULTS

The Measurement of PS Exposure during Apoptosis—PS exposure is an important end point in apoptosis that has not been studied in detail. A decrease in RVV clotting time has been...
used by previous investigators as an indirect measure of PS exposure on the surface of apoptotic cells (4). Jurkat cells treated with anti-Fas mAb (250 ng/ml) showed a significant decrease in plasma clotting time compared with control cells (Fig. 1A), indicating an increase in PS exposure on the cell surface. The clotting assay was sensitive enough to detect the dose-dependent increase in PS exposure induced following stimulation with increasing doses of anti-Fas mAb (10–250 ng/ml, Fig. 1B), which was expressed as the percentage change in clotting time (or PS exposure) from unstimulated controls.

The maximal change in PS exposure achieved with 125–250 ng/ml anti-Fas mAb was 31%. PS:PC liposomes (30:70 mol %), used as a positive control, caused a 48% change in clotting time compared with control liposomes (PC, 100 mol %). PS:PC liposomes at 10:90 mol % and 20:80 mol % caused a 38 and 47% change in clotting time, respectively.

PS Exposure, Blebbing, and Nuclear Fragmentation in Apoptotic Jurkat Cells—The kinetics of PS exposure, plasma membrane blebbing, and nuclear fragmentation were compared in Jurkat cells stimulated by anti-Fas mAb (250 ng/ml, Fig. 2A). PS exposure and plasma membrane blebbing increased at between 30 and 60 min, and both parameters began to plateau thereafter, reaching a maximum at between 90 and 120 min (Fig. 2A). Apoptotic nuclei were detected in 40% of the cells by 60 min and continued to increase until 120 min, at which time 80% of the cells exhibited nuclear fragmentation. At 120 min, the plasma membrane was intact, and secondary necrosis had not occurred (trypan blue permeability was <5%).

PS exposure stimulated by anti-Fas mAb (250 ng/ml) was also quantitated directly by measuring the percentage of cells binding the FITC-labeled annexin V (Fig. 2B). Annexin binding increased to a maximum of 58%, indicating that this proportion of the anti-Fas mAb-stimulated Jurkat cells had exposed PS, a result similar to that reported recently using the same method (64). The cells were also co-stained with propidium iodide to assess plasma membrane integrity, and at 120 min cells remained impermeable to propidium iodide (data not shown), hence secondary necrosis had not occurred.

PS Exposure, Blebbing, and Nuclear Fragmentation in Apoptotic U937 Cells—In U937 cells, the combined treatment of TNF (20 ng/ml) and cycloheximide (1 μg/ml) also induced a relatively rapid apoptotic response. PS exposure and plasma membrane blebbing increased at between 60 and 120 min and reached maximum levels between 120 and 180 min (Fig. 3A). Plasma membrane blebbing decreased as cells began to fragment at 240 min. Apoptotic nuclei were detected in 38% of the cells at 120 min, increasing to 80% by 180 min (Fig. 3A). PS exposure as determined by annexin binding was positive in 6, 32, and 55% of the cell population at 60, 120, and 240 min, respectively (Fig. 3B). The maximum of 55% was similar to that attained in anti-Fas mAb-stimulated Jurkat cells (Fig. 2B). Plasma membrane integrity was maintained, since propidium iodide staining was negative (data not shown). Annexin binding in the unstimulated controls was less than 5% at all time points.

The Effect of Protease Inhibitors on DEVD-AMC Cleavage in Apoptotic Jurkat Cells—The kinetics of DEVD-AMC cleavage in the Fas/Jurkat and TNF/U937 systems was assessed by the cleavage of the fluorogenic substrate DEVD-AMC in cell lysates. DEVD-AMC is a specific substrate for CPP32-like enzymes and represents the cleavage site in poly-(ADP-ribose) polymerase, DNA-PK, U170, and the other zymogen-PI-3 kinase (64). The maximal rate of AMR release over time (V,

The Effect of Protease Inhibitors on DEVD-AMC Cleavage in Apoptotic Jurkat and U937 Cells—Competitive inhibition of the DEVD-AMC activity was performed in vitro by adding...
DEVD-CHO, a specific inhibitor of CPP32-like enzymes (60, 65, 66) at various concentrations (Fig. 5) to lysed anti-Fas mAb-stimulated Jurkat cells and TNF-stimulated U937 cells in the presence of substrate. The percentage change of maximum $V_0$ was assessed in activated Jurkat cells at 45 min and in activated U937 cells at 90 min. DEVD-CHO inhibited the enzyme activity comparably in both cells with an IC$_{50}$ of 30 nM and 60 nM and a maximal inhibition in the nanomolar range. These concentrations are in agreement with other recent studies of ICE-like protease activity (60, 66).

By comparison, the effect of a cell-permeable inhibitor, VAD-cmk, on ICE-like protease activity was tested by preincubation with Jurkat and U937 cells in culture (Table I). VAD-cmk caused a dose-dependent inhibitory effect in both systems, although the anti-Fas/Jurkat response was inhibited to a greater degree than the U937/TNF-mediated response (approximate IC$_{50} = 0.5$ $\mu$m and 1.5 $\mu$m, respectively). Calpeptin was preincubated with U937 and Jurkat cells in a dose range of 10–100 $\mu$g/ml to test the effect on DEVD-AMC cleavage. In comparison with vehicle controls, no significant inhibition of DEVD-AMC cleavage was seen (data not shown).

The Effect of Protease Inhibitors on Anti-Fas mAb-induced Nuclear Fragmentation in Jurkat Cells—The ICE-like protease inhibitor VAD-cmk caused a time- and dose-dependent inhibitory effect on nuclear fragmentation in anti-Fas mAb-stimulated Jurkat cells (Fig. 6, A and B). The approximate IC$_{50}$ was 2 $\mu$m at 120 min (Fig. 6B). The relative lack of inhibitory effect by the calpain inhibitors (calpain inhibitor I and calpeptin) in the same system is also shown (Fig. 6, C and D).

**The Effect of Protease Inhibitors on TNF-induced Nuclear Fragmentation in U937 Cells**—Calpeptin caused a time- and dose-dependent inhibitory effect on nuclear fragmentation in U937 cells stimulated by TNF/cycloheximide at 180 min (approximate IC$_{50}$ = 500 $\mu$m; Fig. 7, C and D). VAD-cmk, however, was not as effective over the same dose range (approximate IC$_{50}$ = 1.20 $\mu$m; Fig. 7, A and B), as it had been in the Jurkat cells.

The Effect of Protease Inhibitors on Anti-Fas mAb-induced PS Exposure, Blebbing, and Fodrin Degradation in Jurkat Cells—Since it has been suggested that the asymmetric distribution of PS in plasma membrane could be at least partly maintained by interaction with cytoskeletal fodrin, one might expect fodrin cleavage would occur with similar kinetics to PS exposure in apoptosis. The cytoplasmic parameters of PS exposure and plasma membrane blebbing occurred with a similar time course to fodrin degradation in Jurkat cells treated with anti-Fas mAb (Fig. 8, A–C). A time-dependent decrease of intact $\alpha$-fodrin (240 kDa) was observed concomitantly with formation of $\sim 150$- and $\sim 120$-kDa breakdown products (Fig. 8C), which have been described previously (47, 67, 68). At 30 min after the addition of anti-Fas mAb, some cleavage was detectable, whereas an almost complete loss of $\alpha$-fodrin was seen by 120 min. The band of 150-kDa fragments was detected prior to 1120-kDa fragments, suggesting that the 120-kDa frag-
ment may be a further breakdown product of the 150-kDa fragment (Fig. 8C).

VAD-cmk (20 μM) inhibited the percentage change in clotting time (PS exposure) and plasma membrane blebbing in Jurkat cells stimulated by anti-Fas, while calpain inhibitor I (60 μg/ml) or calpeptin (100 μg/ml) had no inhibitory effect (Fig. 8, A and B). VAD-cmk significantly protected against α-fodrin cleavage (~70% of the α-fodrin remained intact at 120 min (Fig. 8C). Conversely, calpain inhibitor I (Fig. 8C) and calpeptin (data not shown) did not have any protective effect. The Jurkat/anti-Fas mAb system was also insensitive to calpain inhibitor II (data not shown).

The Effect of Protease Inhibitors on TNF-induced PS Exposure, Blebbing, and Fodrin Degradation in Jurkat and U937 Cells—α-Fodrin cleavage products were also observed in U937 cells treated with TNF, and their formation proceeded with a similar time course relative to PS exposure and blebbing (Fig. 8C). However, for TNF-induced apoptosis in the U937 cell system, the effects of the two classes of protease inhibitors on the cytoplasmic indices PS exposure, blebbing, and α-fodrin cleavage were reversed. The calpain inhibitors were moderately effective at inhibiting PS exposure, blebbing, and α-fodrin cleavage (Fig. 9, A–C). Calpeptin (100 μg/ml) also effectively decreased cellular fragmentation or apoptotic body formation (data not shown). VAD-cmk was a relatively ineffective inhibitor when compared with the Jurkat system stimulated by anti-Fas mAb. Interestingly, VAD did not inhibit the decrease of intact fodrin (240 kDa) to the 150-kDa fragment but appeared to prevent the further degradation of the 150-kDa fragment to the 120-kDa fragment (Fig. 9C). Calpain inhibitor II also had little effect on all parameters tested in the U937/TNF system (data not shown). BOC-aspartyl (benzyl) chloromethylketone, another inhibitor of the ICE family of proteases, produced the same result as VAD-cmk, preferential inhibition in the Jurkat (approximate IC\textsubscript{50} = 20 μM) but not the U937 models at 240 min (data not shown).
Inhibition of HMW DNA Fragmentation—The formation of HMW DNA fragments in TNF-induced apoptosis in U937 cells was inhibited by calpain inhibitor I at 2 h (Fig. 10) but were not affected by VAD-cmk (not shown), in good agreement with other events in TNF-induced apoptosis in U937 cells. Inhibition of ICE-like protease activity has previously been shown to protect from HMW DNA fragmentation in TNF-induced apoptosis in U937 cells.

Is the Differential Inhibition Cell- or Stimulus-specific?—In order to investigate whether the differences in response to protease inhibitors in the Jurkat/anti-Fas mAb and U937/TNF models were due to cell type or stimulus, we investigated U937 cell activation with anti-Fas mAb. VAD-cmk (0.2–20 μM) and calpeptin (1 and 10 μg/ml) showed an inhibitory effect on nuclear fragmentation in these conditions (Fig. 11). The higher (50–100 μg/ml) doses of calpeptin had the propensity to induce apoptosis alone at 6 h (data not shown). We also tested the effects of the protease inhibitors in Jurkat cells stimulated by anti-Fas mAb and cycloheximide and found a similar inhibitory profile with calpain inhibitors or VAD-cmk as in Jurkat cells stimulated by anti-Fas mAb alone (data not shown). Treatment of Jurkat cells with TNF ± cycloheximide did not induce a significant level of apoptosis within 7 h, at which time calpain inhibitor I or calpeptin alone induced apoptosis in unstimulated cells (data not shown); therefore, this combination was not tested.

DISCUSSION

A comparison of the kinetics of plasma membrane blebbing, PS exposure, and fodrin degradation with nuclear fragmentation and the activation of CPP32-like proteases was performed in two apoptotic models. It is not yet known if the extranuclear events occur in series or are linked in some way, and therefore we investigated detailed time courses to observe if one end point occurred before the others. It was not possible to detect which of the events occurred first, apart from the very late event of apoptotic body formation or cellular fragmentation, which occurred after all other events had approached maximum. DEVD-AMC cleavage is an indication of a relatively early event in the signaling cascade, and in the present study we could detect some increase in enzyme activity prior to an increase in extranuclear or nuclear events. However, this could possibly be explained by a relative difference in sensitivity...
between the enzyme assay and the other endpoints measuring changes in cell morphology. In the present study, the kinetics of fodrin degradation were studied in more detail than in the first report of α-fodrin cleavage in apoptosis published recently by Martin et al. (68). Nevertheless, in a comparison of the time sequence of fodrin cleavage with that of plasma membrane blebbing and PS exposure, it was not possible to determine if one event preceded the others. We did find, however, that PS exposure had reached plateau levels in both apoptotic systems before significant cellular fragmentation was observed, indicating that PS exposure may be involved in the initiation of cell fragmentation or apoptotic body formation, as alluded to in early studies of PS exposure and cellular fragmentation in platelets (53–55, 69, 70). Cell fragmentation requires major rearrangement of the cytoskeleton and fusion of opposing cytoplasmic membrane leaflets and, therefore, gross membrane phospholipid rearrangement, such as a local collapse of membrane asymmetry, which occurs during PS exposure (69, 70).

Since the kinetic studies alone were unable to discern which of the extranuclear events occurred first, inhibitor studies with selected protease inhibitors were conducted, since all of the extranuclear events could be linked to plausible substrates of proteases. The rationale for studying calpain involvement was that calpain is a major cytosolic protease that has been reported to play a role in plasma membrane blebbing (48), fodrin degradation (45–47), and possibly PS exposure (71). The calpain inhibitors, calpain inhibitor I and calpeptin, are membrane-permeable peptide aldehydes that are reportedly calpain-specific, although they have been shown to have some inhibitory effect on the cathepsin cysteine proteases (72). The rationale for using inhibitors of the ICE-like proteases, DEVD-CHO and VAD-cmk, was to determine whether the ICE-like proteases regulated all or only some of the extranuclear and nuclear events in TNF/Fas receptor-mediated apoptosis.

Accordingly, we have shown that VAD-cmk inhibited the relatively early event of DEVD-AMC cleavage at similar concentrations in both the Jurkat and U937 systems (Table I), while calpeptin was ineffective in this regard. Nuclear fragmentation was, however, inhibited by both calpeptin and VAD-cmk in a differential manner in the two cell types. In TNF-stimulated U937 cells at 180 min (Fig. 7C) and in Fas-stimulated Jurkat cells at 120 min (Fig. 6A) nuclear fragmentation had reached similar levels of 80%, and therefore a comparison of inhibitors at this point was made. VAD-cmk effectively inhibited nuclear fragmentation in Jurkat cells (IC$_{50}$ = 2 µM, at 120 min; Fig. 6, A and B) but was less effective in U937 cells (IC$_{50}$ > 20 µM at 180 min, Fig. 7, A and B). At this point of comparable nuclear fragmentation in the U937 system,
calpeptin (100 μg/ml) effectively inhibited nuclear fragmentation (Fig. 7, C and D) but did not inhibit, and if anything, slightly potentiated nuclear fragmentation in the Jurkat cells (Fig. 6, C and D). This dose of calpeptin has been shown to completely inhibit the intrinsic calpain activity in platelets (73), but it caused no inhibition of CPP32 (or another CPP32-like protease) activity in U937 cells. This suggests that activation of calpain occurs after the activation of CPP32-like proteases and that calpain may, at best, play a role as an effector rather than an initiator of the apoptotic process, since in the presence of this inhibitor apoptosis will eventually proceed by other mechanisms (Fig. 7C).

The extranuclear events of plasma membrane blebbing, PS exposure, and α-fodrin degradation were also decreased by the calpain inhibitors in U937 cells but not in Jurkat cells (Fig. 6, C and D). This dose of calpeptin has been shown to completely inhibit the intrinsically active calpain activity in platelets (73), but it caused no inhibition of CPP32 (or another CPP32-like protease) activity in U937 cells. This suggests that activation of calpain occurs after the activation of CPP32-like proteases and that calpain may, at best, play a role as an effector rather than an initiator of the apoptotic process, since in the presence of this inhibitor apoptosis will eventually proceed by other mechanisms (Fig. 7C).

Importantly, the two apoptotic systems studied seem to be regulated by different proteases. The inhibitory effect of VAD-cmk on anti-Fas mAb-mediated PS exposure, blebbing, and α-fodrin degradation, as well as DEVD cleavage and nuclear fragmentation in Jurkat cells, indicates that an ICE-like protease plays a role in this apoptotic system. This is in agreement with several recently published reports (24, 35, 74, 75). Alternatively, TNF-induced changes in U937 cells such as PS exposure, blebbing, α-fodrin degradation, and nuclear fragmentation were inhibited by calpain inhibitor I and calpeptin, although they had no inhibitory effect on DEVD-AMC cleavage. VAD-cmk was relatively ineffective at inhibiting extranuclear and nuclear events during the later phases of the apoptotic response in this system. This suggests that calpain may play a more important role in the apparently later apoptotic events induced by TNF in U937 cells. This is in contrast to the reported protection from TNF-induced apoptosis in MCF7 cells with a high expression level of CrmA, an inhibitor of ICE-like proteases (75). It is possible that in MCF7 cells TNF utilizes different proteolytic pathways than in U937 cells. Alterna-
tively, at high concentrations, it is possible that the CrmA-conferred protection is mediated by nonspecific effects on serine proteases (62).

Whether the differential response to protease inhibitors in TNF-stimulated U937 cells and Fas-stimulated Jurkat cells was a cell-specific or a stimulus-specific phenomenon is an important question. It was difficult to satisfactorily answer this question, since it took approximately 6 h for a measurable nuclear fragmentation to occur in U937 cells stimulated with anti-Fas mAb alone. At this time the inhibitors (at least in the higher concentrations) were able to induce apoptosis alone. Nevertheless, in U937 cells stimulated with anti-Fas mAb, calpeptin (1 and 10 μg/ml) was shown to cause some inhibition (Fig. 11), indicating that a cell-specific rather than a stimulus-specific effect was dominant and that both anti-Fas mAb and anti-Fas mAb. At this time the inhibitors (at least in the higher concentrations) were able to induce apoptosis alone. Nevertheless, in U937 cells stimulated with anti-Fas mAb, calpeptin (1 and 10 μg/ml) was shown to cause some inhibition (Fig. 11), indicating that a cell-specific rather than a stimulus-specific effect was dominant and that both anti-Fas mAb and anti-Fas mAb. At this time the inhibitors (at least in the higher concentrations) were able to induce apoptosis alone. Nevertheless, in U937 cells stimulated with anti-Fas mAb, calpeptin (1 and 10 μg/ml) was shown to cause some inhibition (Fig. 11), indicating that a cell-specific rather than a stimulus-specific effect was dominant and that both anti-Fas mAb and anti-Fas mAb. At this time the inhibitors (at least in the higher concentrations) were able to induce apoptosis alone. Nevertheless, in U937 cells stimulated with anti-Fas mAb, calpeptin (1 and 10 μg/ml) was shown to cause some inhibition (Fig. 11), indicating that a cell-specific rather than a stimulus-specific effect was dominant and that both anti-Fas mAb and anti-Fas mAb. At this time the inhibitors (at least in the higher concentrations) were able to induce apoptosis alone. Nevertheless, in U937 cells stimulated with anti-Fas mAb, calpeptin (1 and 10 μg/ml) was shown to cause some inhibition (Fig. 11), indicating that a cell-specific rather than a stimulus-specific effect was dominant and that both anti-Fas mAb and anti-Fas mAb. At this time the inhibitors (at least in the higher concentrations) were able to induce apoptosis alone. Nevertheless, in U937 cells stimulated with anti-Fas mAb, calpeptin (1 and 10 μg/ml) was shown to cause some inhibition (Fig. 11), indicating that a cell-specific rather than a stimulus-specific effect was dominant and that both anti-Fas mAb and anti-Fas mAb. At this time the inhibitors (at least in the higher concentrations) were able to induce apoptosis alone. Nevertheless, in U937 cells stimulated with anti-Fas mAb, calpeptin (1 and 10 μg/ml) was shown to cause some inhibition (Fig. 11), indicating that a cell-specific rather than a stimulus-specific effect was dominant and that both anti-Fas mAb and
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