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Caspases Cleave the Amino-Terminal Calpain Inhibitory Unit of Calpastatin during Apoptosis in Human Jurkat T Cells[1]

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We have previously reported the activation of procalpain μ (precursor for low-calcium-requiring calpain) in apoptotic cells using a cleavage-site-directed antibody specific to active calpain [Kikuchi, H. and Imajoh-Ohmi, S. (1995) *Cell Death Differ.* 2, 195–199]. In this study, calpastatin, the endogenous inhibitor protein for calpain, was cleaved to a 90-kDa polypeptide during apoptosis in human Jurkat T cells. The limited proteolysis of calpastatin preceded the autolytic activation of procalpain. Inhibitors for caspases rescued the cells from apoptosis and simultaneously inhibited the cleavage of calpastatin. The full-length recombinant calpastatin was also cleaved by caspase-3 or caspase-7 at Asp-233 into the same size fragment. Cys-241 was also targeted by these caspases *in vitro* but not in apoptotic cells. Caspase-digested calpastatin lost its amino-terminal inhibitory unit, and inhibited three moles of calpain per mole. Our findings suggest that caspases trigger the decontrol of calpain activity suppression by degrading calpastatin.

Key words: apoptosis, calpain, calpastatin, caspase, Jurkat T-cell.

Apoptosis, a major form of programmed cell death that is essential for normal development and organ homeostasis, involves various intracellular proteolytic systems: caspases (*1–3*), proteasomes (*4*), calpain (*5–10*), and cathepsin (*11, 12*). Among them caspases play pivotal roles in apoptosis-executing processes, since inhibition of caspases results in the suppression of apoptosis in most cells. More than ten species of caspase have been discovered in mammalian cells (*13–16*). Many proteins have been identified as targets for caspases that hydrolyze the carboxyl-terminal-side peptide bond of aspartic acid residues without exception. Caspase-3 preferably recognizes the DXXD sequences found in poly (ADP-ribose) polymerase (PARP) ($DEVD_{213}$) (*17*), α-fodrin ($DETD_{1185}$) (*18, 19*), protein kinase Cδ ($DMQD_{329}$) (*20*), Bcl-2 ($DAGD_{34}$) (*21, 22*), β-catenin ($DLMD_{764}$) (*23*), DNA fragmentation factor 45 (ICAD) ($DETD_{116}$ and $DAVD_{224}$) (*24*), and IκBα ($DRHD_{35}$) (*25*). The cleavage of some substrates is closely related to apoptotic phenomena; the degradation of ICAD results in the activation of DNase and fragmentation of chromosomal DNA (*26 , 27*), and the cleavage of Bcl-2 and IκBα converts these anti-apoptotic molecules into cell death–promoting species (*22, 28*). Caspase-7 resembles caspase-3 in its substrate specificity but shows different profiles of intracellular redistribution during apoptosis (*29*).

On the other hand, the involvement of calpain in apoptosis remains to be confirmed, although calpain activation has been observed during apoptosis in various cells (*5–9, 30*). Previously, we described the activatcion of procalpain μ (a low-calcium-requiring isoenzyme precursor) during apoptosis in human monoblastic U937 cells treated with tumor necrosis factor-α (TNF-α). Furthermore, inhibitors of calpain accelerate cell death, suggesting that calpain contributes to the suppression of apoptosis (*6*).

Based on the findings of *in vitro* experiments, the behavior of calpain in the cell is believed to be as follows. Calpain is synthesized in a precursor form (procalpain) comprising 80-kDa large and 30-kDa small subunits. The large subunit retains the catalytic machinery whose intrinsic activity is restrained by the carboxyl-terminal calcium-binding domains of both subunits at low calcium concentrations. When cells are stimulated and intracellular calcium is mobilized, the carboxyl-terminal domains with calcium ions remove their control on the catalytic domain, and the amino-terminal region of procalpain is autocatalytically cleaved converting the protein to the active form (*31*). The

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Abbreviations: Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-YVAD-cmk, acetyl-Tyr-Val-Ala-Asp-chloromethylketone; Ac-YVKD-CHO, acetyl-Tyr-Val-Lys-Asp-aldehyde; calpain inhibitor I, acetyl-Leu-Leu-norleucine-aldehyde; calpain inhibitor II, acetyl-Leu-Leu-normethionine-aldehyde; calpeptin, carbobenzoxy-Leu-Leu-aldehyde; IPTG, isopropyl-thio-galactopyranoside; mAb, monoclonal antibody; MCA, 4-methyl-coumaryl-7-amide; PARP, poly(ADP-ribose) polymerase; PVDF, polyvinylidene fluoride; TNF, tumor necrosis factor; Z-Asp-CH_2-DCB, carbobenzoxy-Asp-[(2,6-dichlorobenzoyl)oxy] methane; Z-IEAL-CHO, carbobenzoxy-Ile-Glu(OtBu)-Ala-Leu-aldehyde; Z-LLnV-CHO, carbobenzoxy-Leu-Leu-Nva-aldehyde; Z-VAD-fmk, carbobenzoxy-Val-Ala-Asp-fluoromethylketone.

active calpain thus generated catalyzes the hydrolysis of target proteins. Calpastatin, the endogenous inhibitor protein of calpain, may regulate calpain in various states (*32*). Native calpastatin contains three or four calpain inhibitory units. Upon interaction with calpain in the presence of calcium, calpastatin is cleaved into inhibitory units with their calpain-binding ability intact. Calpastatin can inhibit the activation of procalpain as well as the proteolysis of substrates by active calpain.

In apoptotic U937 cells, no more than 1% of procalpain μ is proteolytically converted to the active form as detected by a cleavage-site-directed antibody that specifically binds a proteolyzed polypeptide but not the uncleaved native protein (*6*). It has been difficult to explain this low amount of active calpain in the presence of excess calpastatin in apoptotic cells. In this paper, the caspase-mediated cleavage of calpastatin has been characterized during apoptosis in human T Jurkat cells. Wang *et al.* also reported that calpastatin is cleaved by caspases (*33*), but their observations included errors. We have identified the precise cleavage site and found that the amino-terminal inhibitory unit is broken in calpastatin.

MATERIALS AND METHODS

Reagents—The following reagents were obtained from the following sources: carbobenzoxy-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), Bachem (Switzerland); acetyl-Tyr-Val-Lys-Asp-aldehyde (Ac-YVKD-CHO), acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVKD-cmk), acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO), carbobenzoxy-Asp-[(2,6-dichlorobenzoyl)oxy] methane (Z-Asp-CH_2-DCB), carbobenzoxy-Ile-Glu(OtBu)-Ala-Leu-aldehyde (Z-IEAL-CHO) and carbobenzoxy-Leu-Leu-Nva-aldehyde (Z-LLnV-CHO), Peptide Institute (Osaka); staurosporine, kanamycin and isopropyl-thio-galactopyranoside (IPTG), Sigma Chemicals (St. Louis, MO, USA); calpain inhibitor I (acetyl-Leu-Leu-norleucine-aldehyde) and calpain inhibitor II (acetyl-Leu-Leu-normethionine-aldehyde), Nacalai Tesque (Kyoto). Lactacystin (*34*) and calpeptin (carbobenzoxy-Leu-Leu-aldehyde) (*35*) were kindly donated by Dr. S. Omura (The Kitasato Institute), and Drs. J. Kambayashi, M. Sakon, and K. Fujitani (Osaka University), respectively.

Cell Culture and the Induction of Apoptosis—Human T-lymphoblastoid Jurkat cells (from the Japan Research Resources Bank) were maintained in RPMI1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM Gln, 100 U/ml penicillin G, and 200 μg/ml streptomycin at 37°C under a humidified atmosphere of 5% (v/v) CO_2. For apoptotic induction, the cells (6×10^5 cell/ml) were challenged with 25 ng/ml anti-Fas mAb (MBL, Nagoya), 50 U/ml TNF-α, 0.5 μM staurosporine, 50 μM Z-IEAL-CHO, or 50 μM Z-LLnV-CHO. For microscopic analysis, the cells were cytospun and stained with Carrazi's Hematoxylin as described previously (*36*).

Immunoblotting—The following antibodies were raised in rabbits using synthetic peptides as haptens conjugated with a carrier protein, and purified from antisera by affinity chromatography on immobilized antigen peptides: anti-human calpastatin against residues 680–708 and anti-human PARP against residues 215–224. A cleavage-site-directed antibody for calpain μ, anti-ACTμ, was described by Kikuchi and Imajoh-Ohmi (*6*).

Cells were harvested and washed with 0.15 M NaCl. Trichloroacetic acid was added to the cell suspension to a final concentration of 10% (w/v) to inactivate cellular enzymes, and the sample was placed on ice for 30 min (*37*). Precipitates were recovered by centrifugation (15,000 ×*g* for 5 min in the cold), dissolved in SDS-sample buffer containing 2.3% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, and 10 μg/ml Bromophenol Blue in 125 mM Tris-HCl, pH 6.8, and heated at 100°C for 5 min. Samples were subjected to 7.5% (w/v) acrylamide SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore). The membrane filter was soaked in Tris-buffered saline (0.15 M NaCl in 20 mM Tris-HCl, pH 7.5) containing 20 mg/ml BSA for 1 h at room temperature, incubated with antibodies overnight at 4°C, washed to remove the excess antibodies, and incubated with anti-rabbit IgG conjugated with alkaline phosphatase (Promega, Madison, WI, USA). Antigens were visualized by the enzymatic reaction of alkaline phosphatase with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium.

Preparation of Recombinant Calpastatin—Recombinant calpastatin was expressed in *Escherichia coli* strain BL21(DE3)pLysE and purified as described previously (*38*). In brief, bacteria carrying the expression plasmids were cultured in L-broth containing 0.1% (w/v) glucose and 30 μg/ml kanamycin at 37°C to middle log phase, and treated with 1 mM IPTG for 3 h. The cells were collected by centrifugation, disrupted by freezing and thawing in buffer A (50 mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 5 mM 2-mercaptoethanol), and boiled for 10 min. The heat-resistant proteins were concentrated with ammonium sulfate at 55% saturation. The pellets were dissolved in buffer A, applied to a DEAE-Sepharose CL-6B (Pharmacia) column, and eluted with buffer A containing 0.1 M NaCl. The purified recombinant calpastatin appeared as a single band with an apparent molecular mass of 120 kDa on SDS-PAGE.

The amino-terminally deleted calpastatin (residues 234–708) corresponding to the 90-kDa fragment was amplified by PCR from the full-length calpastatin cDNA and subcloned into a bacterial expression vector pET-15b (Novagen, Madison, WI, USA). The oligonucleotides used for PCR primers were 5'-CCTCCTAGCCATGGCCTTGTCATCTGACTTCAC-3' (5'-primer) and 5'-AAGGTATCTGGTATCTGCATGT-3' (3'-primer). Expression and purification of the recombinant protein were performed as for the full-length calpastatin except that the host cells used were *E. coli* strain BL21(DE3).

In Vitro Cleavage of Calpastatin—Jurkat cells were cultured with anti-Fas mAb for 8 h, and the cytosolic fraction was prepared by freezing and thawing as described by Harvey *et al.* (*39*). Recombinant calpastatin was incubated with apoptotic cytosol for 2 h at 37°C. The reaction was stopped by the addition of an equal volume of 2 × SDS-sample buffer, and the sample was heated for 5 min at 100°C.

Amino-Terminal Sequencing—Recombinant calpastatin was incubated with caspase-7 (MBL, Nagoya) for 24 h at 37°C. The digests were subjected to SDS-PAGE and transferred onto a PVDF membrane. The proteins were stained with 0.1% (w/v) Coomassie Brilliant Blue R250 in methanol, and the bands were excised and analyzed with a Beckman LF3000 protein sequencer.

Other Methods—The casein-hydrolyzing activity of cal-

pain was examined as described previously (*40*). Proteins were determined by staining with Coomassie Brilliant Blue in SDS–polyacrylamide gels.

RESULTS

Cleavage of Calpastatin and Activation of Procalpain μ during Fas-Mediated Apoptosis—A cytotoxic anti-Fas monoclonal antibody (mAb) induced nuclear fragmentation in human T-lymphoblastoid Jurkat cells, a morphological feature typical of apoptosis (Fig. 1). When nuclei were stained with hematoxylin, apoptotic cells could be clearly identified by light microscopy. Apoptotic cells appeared several hours after induction with the mAb, and increased to more than 80% in one day. Calpastatin was characterized by immunoblotting in cells undergoing apoptosis (Fig. 2A). Under non-apoptotic conditions, Jurkat cells expressed a four-inhibitory-unit type of calpastatin exhibiting a molecular size of 120 kDa on SDS-PAGE. When apoptosis was induced with anti-Fas mAb, a 90-kDa polypeptide, presumably related to calpastatin, was detected by an antibody raised against the carboxyl-terminal region of human calpastatin. The 90-kDa fragment was seen 4 h after apoptotic induction, and native calpastatin was hardly detected by 12 h. We also examined the proteolysis of poly(ADP-ribose) polymerase (PARP), a nuclear target for caspase-3 and/or-7, which cleave PARP at Asp-214 to generate a 30-kDa DNA-binding amino-terminal and 85-kDa catalytic carboxyl-terminal fragments. As shown in panels A and B in Fig. 2, the cleavage of calpastatin occurred in parallel with that of PARP. On the other hand, an active form of calpain μ was observed from 12 to 24 h (Fig. 2C). The active calpain was visualized by a cleavage-site-directed antibody specific for the amino-terminal region of the catalytic subunit of calpain μ activated by autolysis (*6*). The degradation of calpastatin apparently preceded the proteolytic activation of procalpain.

To identify proteases that catalyze the degradation of calpastatin, we first examined the effects of protease inhibitors on the cleavage of calpastatin. Inhibitors of calpain, calpeptin, calpain inhibitor I and calpain inhibitor II, did

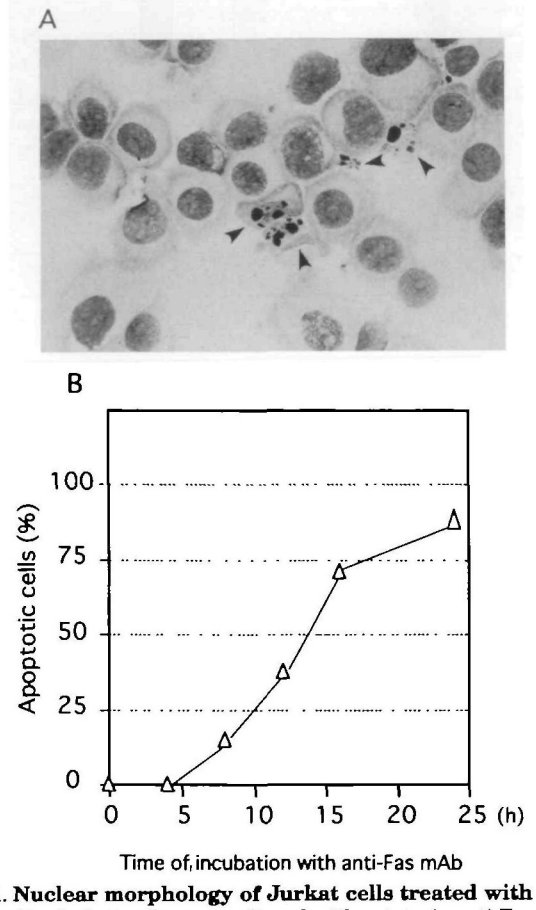

Fig. 1. **Nuclear morphology of Jurkat cells treated with anti-Fas mAb.** Jurkat cells were cultured with cytotoxic anti-Fas mAb, mounted after different times on a slide glass by cytocentrifugation, and stained with Carrazi's Hematoxylin (Muto Pure Chemicals, Tokyo) as described previously (*36*). Nuclei were condensed into fragments (arrowheads in panel A) when cells undergo apoptosis. Apoptotic cells were counted under a light microscopy (B).

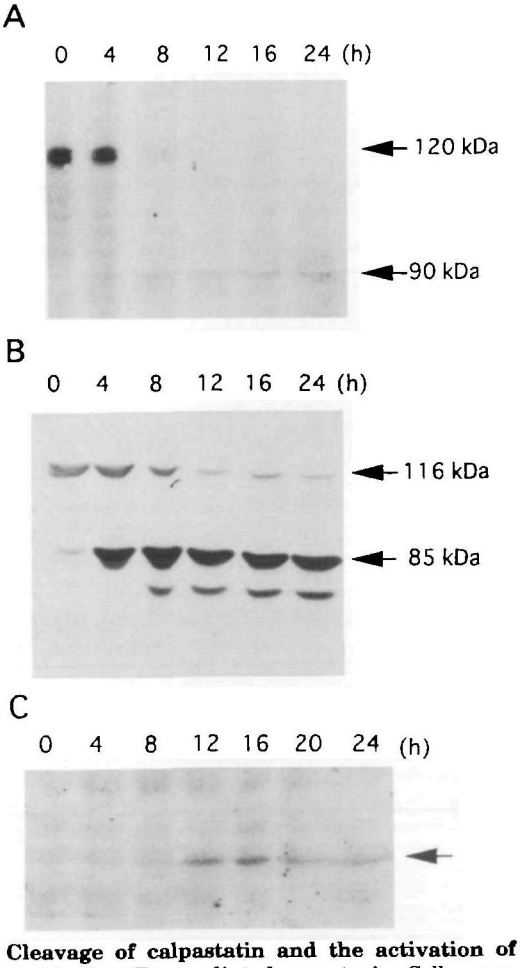

Fig. 2. **Cleavage of calpastatin and the activation of procalpain μ during Fas-mediated apoptosis.** Cells were challenged with anti-Fas mAb, and sampled for immunoblotting at the indicated times (0 to 24 h). Native calpastatin (120 kDa) and its fragment (90 kDa) were stained with an anti-peptide antibody raised against the carboxyl-terminal 23-residue region of human calpastatin (arrows in panel A). PARP (116 kDa) and its caspase-catalyzed fragment (85 kDa) stained with an anti-PARP antibody against residues 215–224 of human PARP as indicated by arrows in panel B. The activation of procalpain was detected by a cleavage-site-directed antibody for human calpain μ (panel C, arrow).

not inhibit Fas-mediated apoptosis of Jurkat cells (Fig. 3A). Neither of these inhibitors affected the proteolysis of calpastatin and PARP during apoptosis (Fig. 3, B and C). In the presence of calpain inhibitors, however, the activation of procalpain was suppressed (Fig. 3D). Lactacystin, a proteasome-specific inhibitor, did not inhibit calpain activation, although it accelerated apoptosis and the proteolysis of calpastatin and PARP to some extent. Next, we investigated the effects of inhibitors of various caspases on apoptosis and proteolysis (Fig. 4). Z-Asp-CH_2-DCB and Z-VAD-fmk, caspase inhibitors with broad specificities, completely suppressed apoptosis and the proteolysis of calpastatin and PARP. Ac-DEVD-CHO, which inhibits caspase-3/caspase-7 and suppresses apoptosis, also prevented the degradation of calpastatin. However, Ac-YVAD-cmk and Ac-YVKD-CHO, which inhibit caspase-1 and are less effective against Fas-mediated apoptosis of Jurkat cells, did not prevent the generation of the 90-kDa fragment from calpastatin.

In Vitro Proteolysis of Calpastatin by Caspases and the Identification of Cleavage Sites—A recombinant human calpastatin was prepared and incubated with cytosolic fractions from Jurkat cells. When calpastatin was mixed with normal cytosol, it was not proteolyzed (Fig. 5A, lane 2). However, calpastatin was broken down to double bands of 90- and 80-kDa during incubation with apoptotic cytosol prepared 8 h after induction (Fig. 5A, lane 4). Recombinant

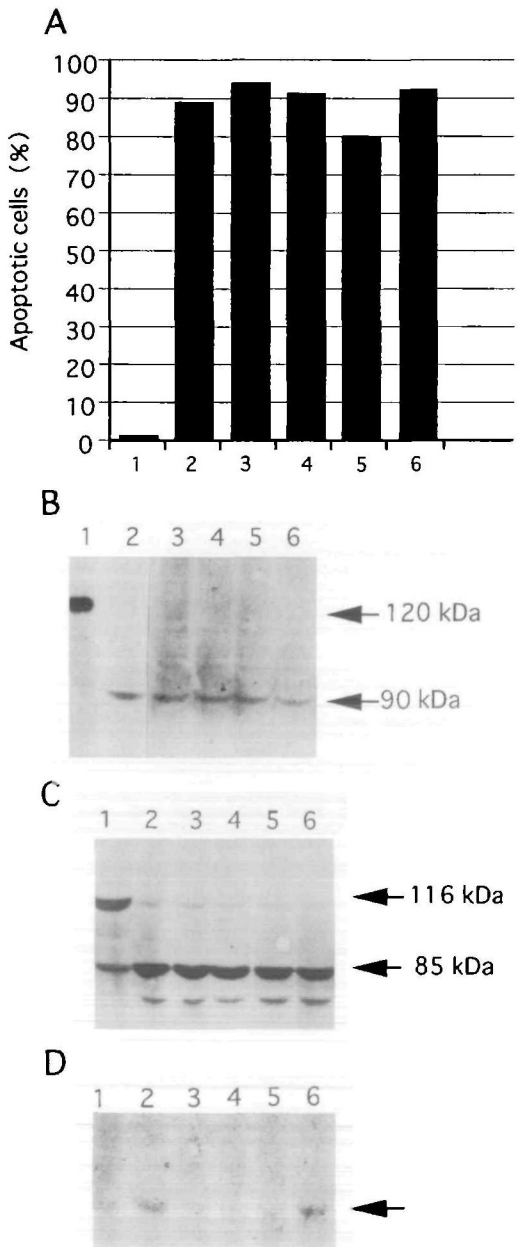

Fig. 3. **Effects of inhibitors of calpain and proteasomes on apoptosis and calpastatin cleavage.** After pretreatment with inhibitors for 3 h, cells were treated with anti-Fas mAb for 24 h. Apoptotic cells (A), cleavage of calpastatin (B), and PARP (C), and the activation of procalpain (D) were examined as described in the legend to Fig. 1. 1, control cells without mAb. Inhibitors added were: 2, none; 3, 100 μM calpeptin; 4, 10 μM calpain inhibitor I; 5,10 μM calpain inhibitor II; and 6, 50 μM lactacystin.

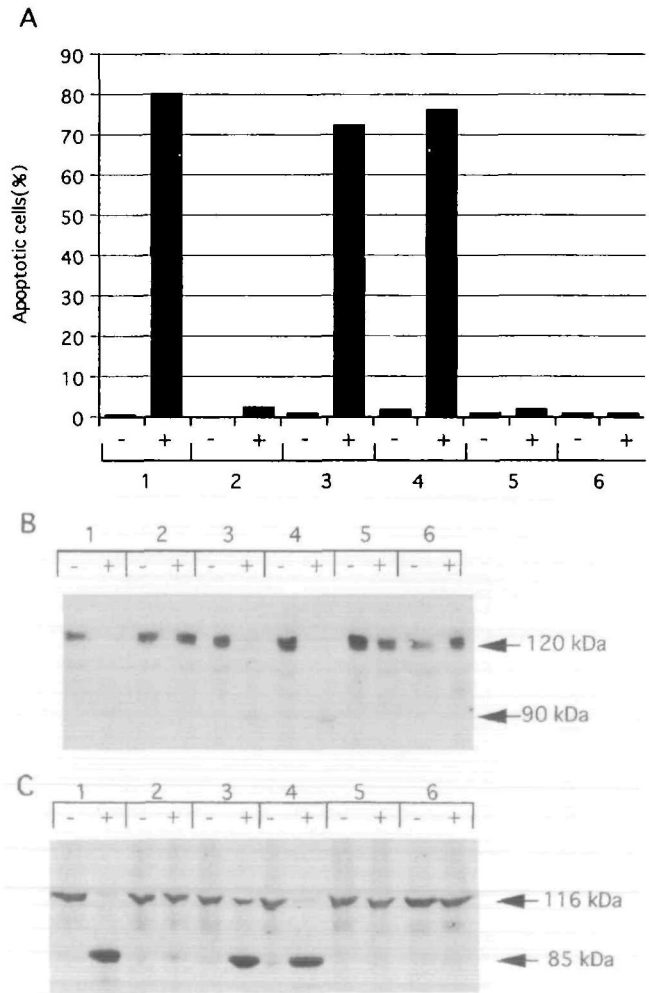

Fig. 4. **Effects of caspase inhibitors on apoptosis and calpastatin cleavage.** Cells were pretreated with caspase inhibitors, and divided into two cultures. Each culture was further incubated with or without anti-Fas mAb for 24 h. Apoptotic cells (A), cleavage of calpastatin (B) and PARP (C) were examined as described in the legend to Fig. 1 except that the results are shown in the presence (+) or absence (–) of anti-Fas mAb. 1, control cells without inhibitor. Inhibitors (100 μM) added were: 2, Z-Asp-CH_2-DCB; 3, Ac-YVAD-cmk; 4, Ac-YVKD-CHO; 5, Z-VAD-fmk; and 6, Ac-DEVD-CHO.

caspase-3 or caspase-7 also cleaved calpastatin in a similar manner (Fig. 5A, lane 5 and 6). The proteolysis of calpastatin in apoptotic cytosol was abolished by Z-Asp-CH_2-DCB, Z-VAD-fmk, and Ac-DEVD-CHO, but not by Ac-YVAD-cmk (Fig. 5B). The inhibition profiles of the five inhibitors were similar to those of *in vivo* experiments when calpastatin was incubated with recombinant caspase-3 or caspase-7 instead of the cytosol from apoptotic cells (data not shown).

Next, bacterially expressed and purified calpastatin was digested with caspase-7, electrophoresed and blotted on a PVDF membrane, and the two bands were separately applied to a protein sequencer. As shown in Table I, two sites were determined for cleavage by caspase-7. The amino-terminal sequence of the 90-kDa fragment was ALSSDFT, indicating that the cleavage residue is Asp-233. Unexpectedly, the P1 residue of the other site was Cys-241, since the amino-terminus of the 80-kDa fragment was identified as Gly-242. The calpastatin-derived fragment generated in apoptotic cells seems to correspond to the Asp-233–cleaved fragment from recombinant calpastatin as judged by their electrophoretic mobilities. Using an antibody for a region near the cleavage site, it was confirmed that calpastatin is cleaved at Asp-233 in apoptotic cells. Even when the amount of the SDS-PAGE sample was increased, however, the 80-kDa fragment was not detected in dying cells, suggesting that the proteolysis of calpastatin at Cys-241, if it

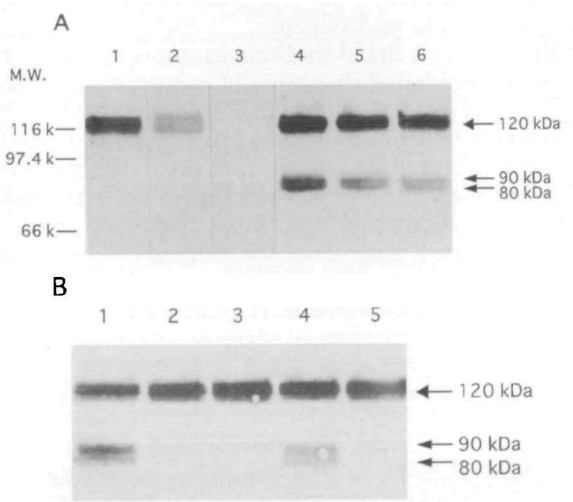

Fig. 5. ***In vitro* cleavage of calpastatin.** A: Recombinant calpastatin was prepared and incubated with the cytosol from Jurkat cells undergoing apoptosis, caspase-3, or caspase-7 as described under "MATERIALS AND METHODS." Staining in immunoblotting was performed using an anti-calpastatin antibody. 1, recombinant calpastatin; 2, non-apoptotic cytosol with calpastatin; 3, apoptotic cytosol without calpastatin; calpastatin was incubated with apoptotic cytosol (lane 4), caspase-3 (lane 5), or caspase-7 (lane 6). Molecular weights of marker proteins are indicated on the left. In panel B, calpastatin was incubated with apoptotic cytosol in the absence (lane 1) or presence of caspase inhibitors: 2, Z-Asp-CH_2-DCB; 3, Z-VAD-fmk; 4, Ac-YVAD-cmk; and 5, Ac-DEVD-CHO.

TABLE I. **Amino-terminal sequences of calpastatin fragments cleaved by caspase-7.**

Fragment	Amino-terminal sequence	Cleavage site
90 kDa	ALSSDFTX*GS	$DAID_{233}$*ALSSDFTCGS
80 kDa	GSPTAAGKKT	$DFTC_{241}$*GSPTAAGKKT

*Not determined.

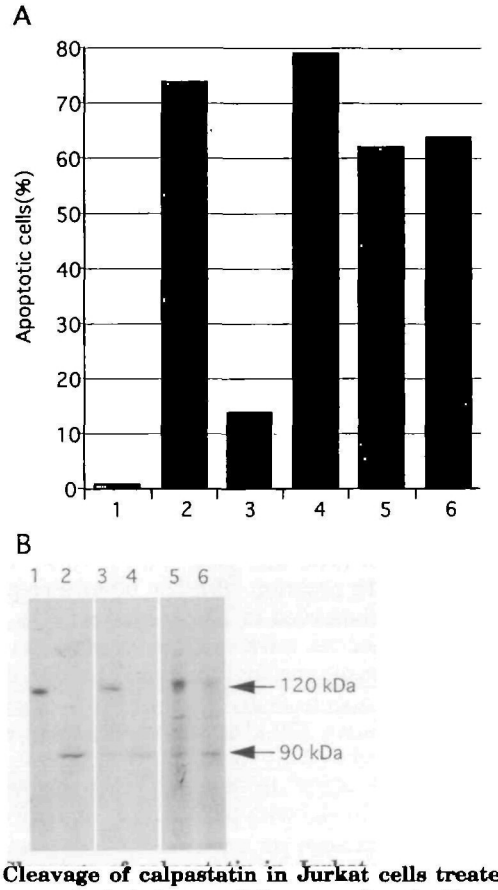

Fig. 6. **Cleavage of calpastatin in Jurkat cells treated with various apoptosis inducers.** Cells were cultured with apoptosis-inducing reagents for 24 h, and apoptotic cells (panel A) and the proteolysis of calpastatin (panel B) were examined as described in the legend to Fig. 1. Apoptosis inducers used were: 1, none; 2, anti-Fas mAb; 3, TNF-α; 4, staurosporine; 5, Z-IEAL-CHO; and 6, Z-LLnV-CHO. In panel B, native calpastatin (120 kDa) and its fragment (90 kDa) are indicated by arrows.

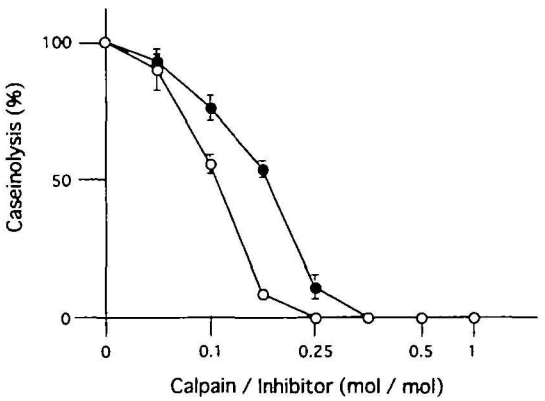

Fig. 7. **Titration of calpastatin activity by calpain.** The caseinolytic activity of calpain was measured in the presence of various amounts of native (○) and cleaved (●) forms of calpastatin. The horizontal axis represents the molar ratio of calpastatin to calpain. The data represent the average of three experiments, and error bars indicate standard deviation.

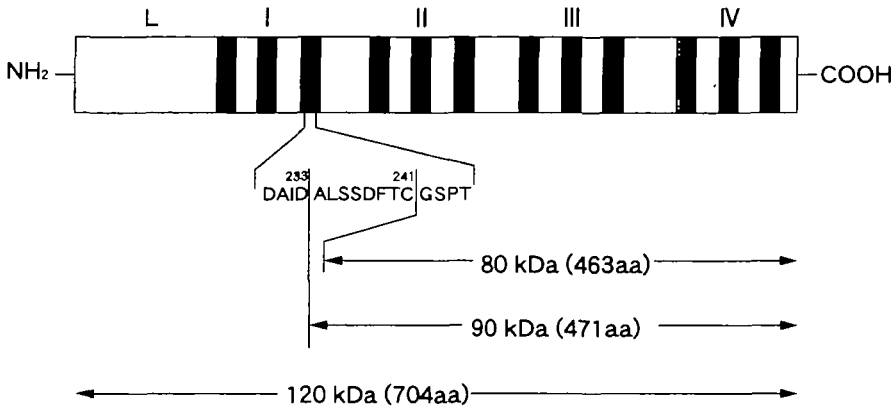

Fig. 8. **Schematic structure of calpastatin.** Non-erythroid calpastatin contains four inhibitory units (domains I to IV) amino-terminally franked by an L domain of unknown function. Each inhibitory unit spans approximately 140 residues with a central inhibitory sequence [subdomain B (■)] between two α-helical regions [subdomains A (▥) and C (▤)]. Subdomains A and C bind to the calcium-binding regions of calpain but do not have inhibitory activity by themselves. Sites cleaved by caspase-3/caspase-7 are indicated and the lengths of the resulting fragments are expressed in molecular mass as well as residue number.

occurs at all, is a minor event *in vivo* (data not shown).

Proteolysis of Calpastatin in Apoptosis Induced by Various Reagents—Next, we examined whether or not calpastatin is cleaved in a similar manner in Jurkat cells when other apoptosis inducers are used (Fig. 6). Jurkat cells express receptors for TNF-α (*41*). TNF-α also induces apoptosis in Jurkat cells, but to a lesser extent, probably because nuclear factor κB is activated and the survival signal overcomes the death program (*42*). The 90-kDa calpastatin fragment was also observed in TNF-α–treated cells, but its proteolysis was not so advanced as in anti-Fas mAb–treated cells. Staurosporine, an inhibitor of protein kinases, effectively induces apoptosis in Jurkat cells where calpastatin is degraded. Native 120-kDa calpastatin was not seen, but the 90-kDa band was also faint, suggesting that further breakdown might occur in staurosporine-treated cells. When cells were cultured with proteasome inhibitors, apoptotic cell death was seen as reported for U937 cells (*36*), and the calpastatin fragment was observed simultaneously.

Inhibitory Activity of Cleaved Calpastatin—Finally, calpain inhibition of the 90-kDa fragment was characterized *in vitro*. Since it was difficult to recover the calpastatin fragment quantitatively from apoptotic cells, we made a cDNA construct for amino-terminally deleted calpastatin (residues 234–708), and expressed the truncated calpastatin in *E. coli*. The electrophoretic mobility of the truncated calpastatin was similar to that of the fragment in apoptotic cells and the 90-kDa fragment generated by caspases (data not shown). The specific activities of the native and truncated calpastatins were 3,600 and 3,750 U/mg protein, suggesting that they retain four and three inhibitory units, respectively. In fact, as shown in Fig. 7, the truncated calpastatin inhibited the caseinolytic activity of three molecules of calpain. We saw no decrease in the affinity between the truncated calpastatin and calpain under the conditions used.

DISCUSSION

The activation of calpain has been observed during apoptosis in various cells: T cell receptor-mediated cell death in 2B4 murine T cell hybridoma and activated lymphocytes (*43*): dexamethasone-treated mouse thymocytes (*5*); human monoblastic U937 cells treated with TNF-α (*6*); serum-depleted human fibroblast TIG-3 cells (*30*); and camptothecin-treated human promyeloid HL-60 cells (*44*). Although an increase in intracellular calcium is observed in some apoptotic cells (*45*, *46*), the low levels of calcium mobilization should not be sufficient to activate procalpain. Moreover, calpastatin may suppress the activation of procalpain. To our knowledge, the active calpain level is at most 1% that of cellular procalpain when quantified using a cleavage-site-directed antibody against the active calpain subunit (*6*). If calpastatin is at the normal level comparable to that of procalpain, it should completely inhibit the catalytic activity of the activated calpain.

In this study, we found that calpastatin is cleaved during apoptosis. Considering that procalpain is activated in apoptotic cells and that calpastatin undergoes degradation upon interaction with active calpain, we first supposed that calpastatin is digested by calpain and binds the enzyme to modulate its activity in apoptotic cells. This hypothesis is ruled out by the finding that the activation of procalpain apparently occurs later than the cleavage of calpastatin. Moreover, the inhibition of calpain activity does not prevent the degradation of calpastatin. Thus, calpain does not seem to be involved in the cleavage of calpastatin even as a trigger. In fact, calpastatin is cleaved by calpain in a calcium-dependent manner with a profile that differs from that in dying cells (data not shown).

The cleavage is mediated by caspases because pan-caspase inhibitors, but not other protease inhibitors strongly prevent proteolysis *in vivo* and *in vitro*. The most likely protease appears to be caspase-3/caspase-7, since bacterially expressed calpastatin is digested *in vitro* by recombinant caspase-3 and caspase-7 to fragments similar to those formed in apoptotic cells. Furthermore, the cleavage occurs after the P_1 aspartate in DAID233/A, which fits DXXD, the consensus motif for caspase-3/caspase-7 (*47*). The cleavage site sequence DAID/A is conserved among calpastatins from human (*48*), porcine (*49*), bovine (*50*), sheep (accession number: U66320), and rabbit (*51*), while the P_4 position Asp in rat (*52*) and mouse (*53*) calpastatins is replaced by His and Gln, respectively. The caspase-mediated cleavage of calpastatin is expected to occur in apoptotic cells from various mammals.

In the case of calpastatin, it is difficult to identify the cleavage sites from electrophoretic mobilities of the resulting fragments on SDS-PAGE. Calpastatin moves more slowly in SDS–polyacrylamide gels than expected from its molecular size. For example, erythrocyte calpastatin, whose amino-terminus is Ser-287 and retains three inhibitory

units, migrates similarly to BSA (70 kDa), although its molecular weight is calculated to be 46,000 based on its amino acid sequence (*54*). The calpastatin-derived polypeptide observed in apoptosis is apparently longer than erythrocyte calpastatin. Thus, the amino terminus should be located around residues 230–240. In fact, amino-terminal sequence analysis of *in vitro* digested recombinant calpastatin revealed one cleavage site by caspases to be DAID-233/A, the preferred sequence for caspase-3 and caspase-7. On the basis of its primary structure, non-erythrocyte-type human calpastatin harbors five DXXD motifs, DLID162/T, DAID233/A, DPED450/G, DALD572/K, and DPID652/A. The latter three DXXDs appear not to be recognized by caspases, since further calpastatin digestion was not detected by immunoblotting.

Wang *et al.* recently reported the fragmentation of calpastatin by caspase-1 and -3 during apoptosis in Jurkat and human neuroblastoma cells (*33*). Using truncated forms of recombinant calpastatin, they assigned three positions for cleavage by caspase-1, ALDD159/L, LSSD238/F, and ALAD439/S (residue numbers are aligned with our criterion). Caspase-3 shares the Asp-238 site, generating a 75-kDa fragment that presumably corresponds to the DAID-233-cleaved 90-kDa polypeptide in our experiments. Wang *et al.* failed to observe Asp-233 cleavage by either of caspase-1 and-3. Although we have not examined whether caspase-1 cleaves calpastatin directly, it is not likely that caspase-1 participates in the limited proteolysis of calpastatin, at least in Jurkat cells, for the following reasons. First, the degradation of calpastatin is not suppressed by inhibitors of caspase-1, Ac-YVAD-cmk, and Ac-YVKD-CHO. Second, when Jurkat cells are treated with anti-Fas antibody, the acetyl-DEVD-4-methyl-coumaryl-7-amide (MCA)–hydrolyzing activity increases in the cytosol within a few hours, suggesting that active caspase-3/caspase-7 is generated. However, 7-amino-4-methylcoumarin is not released from acetyl-YVAD-MCA, a substrate for caspase-1 (data not shown). Finally, apoptosis of Jurkat cells is not prevented by caspase-1 inhibitors. These observations indicate that caspase-1 is not involved in the Fas-mediated apoptosis of Jurkat cells, which probably applies to other apoptosis inducers tested in this study.

Curiously, another site, DFTC241/G, is recognized by recombinant caspase-3/caspase-7. This is the first report that caspases recognize a P_1 residue other than aspartic acid. Hydrolysis at this position is not due to contaminating proteases, if any, because caspase inhibitors suppress the cleavage simultaneously with the cleavage after DAID233. Since the calpastatin has undergone heat treatment during preparation, cysteine residues might be oxidized to cysteic acid. Caspases may mistake the cysteic acid for Asp, a supposition that remains to be experimentally confirmed. It is not likely that such a modification occurs *in vivo*, since calpastatin is broken down to a 90-kDa single band, corresponding to cleavage after DAID233, in apoptotic Jurkat cells. However, our finding suggests the possibility that cleavage sites can be newly generated by post-translational modifications; for example, the phosphorylation that has been reported for the P_1' position Ser for caspase-3 in IκBα (*25*).

Finally, we discuss the biological significance of calpastatin degradation during apoptosis. As shown in Fig. 8, calpastatin contains four calpain inhibitory units (domains I to IV, numbered from the amino terminus) (*55–57*). Each unit spans approximately 140 residues and is further divided into three subdomains A, B, and C. The central subdomain B has a conserved sequence, TIPPXYR, and binds the catalytic region of calpain (*58–61*). On the other hand, subdomains A and C, which form α-helices, interact with the calcium-binding domains of calpain, probably reinforcing the inhibition by subdomain B (*62–65*). When calpastatin binds active calpain in the presence of calcium, the domains are cut off with their calpain-binding ability intact (*66*, *67*). The calpain-mediated cleavage of calpastatin occurs between domains. The translocation and catalysis of calpain may be thus modulated by the calpastatin domains (*68*).

As shown in Fig. 2A, the amount of 90-kDa calpastatin fragment is lower than expected from the reduction in molecular mass. The fragment apparently undergoes further degradation by some proteases not yet identified. Cleavage by caspases may be a signal for instability. In fact, the caspase-catalyzed fragment of calpastatin is not observed by immunoblotting when the apoptotic cytosol is frozen and thawed prior to electrophoresis in SDS-containing buffer.

The cleavage site is located in subdomain C in the amino-terminal inhibitory unit (domain I). Therefore, the inhibitory activity of domain I should be decreased by the cleavage. As far as we examined, the carboxyl-terminal three inhibitory units were effective calpain inhibitors after cleavage. However, domain I is reported to show the strongest inhibition of calpain among the four domains (*57*). Thus, the overall activity of calpastatin in the cell should be considerably decreased by the cleavage. We have previously reported that active calpain possibly down-regulates apoptosis in dying cells (*6*). A recent finding by Wood *et al.* that calpain digests proapoptotic protein Bax during apoptosis (*44*) supports our proposal. In any case, calpain is unlikely to participate in a proapoptotic pathway. The cleavage of calpastatin is expected to lead to various events including the activation of procalpain, enhancement of the catalytic activity of activated calpain, and modulated translocation of calpain (*69*). Caspase has been established as an apoptosis-executing protease. However, calpain remains to be investigated for its role in apoptosis. This report provides evidence for a linkage between the two proteolytic systems, and may lead to a breakthrough in understanding the physiological significance of calpain.

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