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Caspase-Mediated Fragmentation of Calpain Inhibitor Protein Calpastatin during Apoptosis

Kevin K. W. Wang,*,[1] Rand Posmantur,*,† Ravi Nadimpalli,* Rathna Nath,* Panaiyur Mohan,‡ Ralph A. Nixon,‡ Robert V. Talanian,§ Martha Keegan,§ Linda Herzog,§ and Hamish Allen§

*Laboratory of Neuro-biochemistry, *Department of Neuroscience Therapeutics, †Department of Immunopathology, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, Michigan 48105; ‡Nathan Kline Institute for Psychiatric Research, New York University Medical Center, Department of Psychiatry, 140 Old Orangeburg Road, Orangeburg, New York 10962; and §BASF Bioresearch Corporation, 100 Research Drive, Worcester, Massachusetts 01605*

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Two cysteine protease families (caspase and calpain) participate in apoptosis. Here we report that the endogenous calpain inhibitor calpastatin is fragmented by caspase(s) to various extents during early apoptosis in two cell types. In anti-fas or staurosporine-treated Jurkat T-cells, the high-molecular-weight form (HMW) of calpastatin (apparent M_r 110 K) was extensively degraded to immunoreactive fragments of M_r 75 K and 30 K In apoptotic SH-SY5Y human neuroblastoma cells, HMW calpastatin was degraded to a major immunoreactive fragment of 75 K. In both cell types, fragmentation of HMW calpastatin was blocked by a caspase-specific inhibitor carbobenzoxy–Asp–$CH_2OC(O)$–2,6-dichlorobenzene. *In vitro* translated HMW calpastatin was sensitive to proteolysis by recombinant caspase-1, -3, and -7. By contrast, *in vitro* translated LMW calpastatin (which lacks domains L and I) was cleaved into multiple fragments only by caspase-1 and was relatively resistant to caspase-3, -7, and other caspases tested. Consistently with that, purified erythroid LMW calpastatin was also highly susceptible to caspase-1 digestion. Recombinant human calpastatin spanning domain I through III (CAST(DI–III)) was found cleaved by caspase-1 at at least three sites, located in either the A or the C helix of domains I and III ($ALDD_{137}$*L, $LSSD_{203}$*F and $ALAD_{404}$*S), while only a single site ($ALDD_{137}$*L) was cleaved by caspase-3. These findings suggest that both HMW and LMW calpastatins are more vulnerable to caspase-1 than to caspase-3. Surprisingly, both erythroid LMW calpastatin and recombinant CAST(DI–III) fragmented by caspase-1 suffered only a less than twofold reduction of inhibitory activity toward calpain. We propose that the proteolysis of calpastatin in early apoptosis might have yet unidentified effects on the cross-talk between the two protease systems. © 1998 Academic Press

Key Words: **apoptosis, cell death, protease inhibitor, necrosis, caspase, calpain, calpastatin, CPP32, ICE.**

Apoptosis (or programmed cell death) is a form of cell death that occurs physiologically as well as pathologically (1–3). It appears that protease activation plays a critical role for the progression of apoptosis (4). The involvement of the interleukin-1beta converting enzyme (ICE)/caspase family has been clearly demonstrated in a variety of systems (see Ref. 4). Currently, 10 members of this family have been cloned. Human caspase-1 (interleukin converting enzyme, ICE), has a broad distribution (5, 6) but is not expressed in all cell types (7, 8). Overexpression of ICE can lead to early apoptosis in cultured cells (9). However, analysis of ICE-knockout transgenic mice indicated that ICE does not have an autonomous role in apoptosis, except possibly for Fas-induced apoptosis in thymocytes (10, 11). The substrate specificity for caspase-1 is rather limited. Pro-IL-1beta and Interferon-gamma inducing factor (IGIF, IL-18) are the only convincingly demonstrated endogenous substrates (12–14). Human caspase-3 (CPP32), however, is a proapoptotic protein that is distributed in all cell types (7). Caspase-3 is capable of cleaving poly(ADP-ribose) polymerase (PARP), α-spectrin, PKC-delta, and a growing number of other proteins that are found cleaved in apoptosis *in situ* (14–16). Caspase-3 knockout mice have a major defect in

[1] To whom correspondence should be addressed. Fax: (734) 622-7178. E-mail: kevin.wang@wl.com. Correspondence may also be addressed to Dr. Hamish Allen. Fax: (508) 754-6742.

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machinery of neuronal apoptosis (11). Calpain, on the other hand, has been reported to be activated during apoptosis in T-cells, thymocytes, and cerebellar granular neurons and neuroblastoma SH-SY5Y cells (17–20). Interestingly, α-spectrin is apparently dually cleaved by both calpain and a caspase-3-like activity in apoptosis (19). Both caspase and calpain are of the cysteine protease family. While calpain requires calcium for activity, caspase activity is unaffected by calcium ion. So far, no direct linkages have been found between the two protease systems.

Calpastatin is a well-documented specific endogenous inhibitor for calpain. Although only one gene (located in chromosome 5) for calpastatin was identified (21), several mature forms of calpastatin exist. The full-length form has domain L and four repeating inhibitory domains (I–IV) with a calculated mw of 68 kDa, but displays anomalous high apparent M_r of 105–110 K on SDS–PAGE) (20, 21) (Fig. 2). There is another posttranslationally produced form found in erythrocytes that lacks the first two domains (L and I) (46 kDa, apparent M_r 68 K on SDS–PAGE) (22). Within each repeated domain, there are three conserved regions (A, B, C). The helical regions A and C apparently interact with the EF-hand calcium binding domains of calpain (IV and VI), respectively (24, 25). Region B, however, apparently interacts directly with the catalytic site of calpain. In fact, 27- and 20-mer peptides based on the core sequence of region B have been found to have inhibitory activities (26, 27). In many cell types, calpastatin is in excess of calpain (28). Thus, it has been suggested that the level of calpastatin can regulate the activity of calpain *in situ*. Calpastatin can be fragmented by calpain *in vitro* but it is not known whether this occurs physiologically (29–30). In this study, we report a novel finding that the HMW form of calpastatin is fragmented to a different extent during early apoptosis in Jurkat T-cells and SY5Y cells. Our evidence indicates that caspase-1 and caspase-3 are responsible for different cleavages. We suggest that calpastatin degradation might be a novel link between the two protease systems.

MATERIALS AND METHODS

Apoptosis in Jurkat t-cells and neuroblastoma SH-SY5Y cells. Human Jurkat T cells (clone JE6-1) was obtained from ATCC (Rockville, MD) and maintained in RPMI-1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 50 U/ml penicillin G, and 50 μ g/ml streptomycin. Jurkat cells were washed once in serum-free medium and suspended at 2×10^6 cell/ml for testing. Five hundred microliters of cell suspension was added to each well of 12-well culture plates, followed by the addition of 500 μ L of 1 μ M staurosporine (Calbiochem, La Jolla, CA) and 300 ng/mL anti-Fas (Upstate Biotechnology Inc., Lake Placid, NY) in RPMI medium. Cells were maintained at 37°C in a 5% CO_2 atmosphere and harvested at 3 or 6 h for protein extraction.

Human neuroblastoma SH-SY5Y cells (SY5Y) were grown on 12-well plates to confluency (about 2 million/well) with Dulbecco's modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum, 100 unit/mL penicillin, 100 μ g/mL streptomycin, and 2.5 μ g/mL Fungizone (amphotericin B). Cultures were washed three times with serum-free MEM. Calpain inhibitor I (CalpInh-I; a.k.a. Ac–Leu Leu–Nle–H) (Calbiochem) or carbobenzoxy–Asp–CH_2OC(O)–2,6-dichlorobenzene (Z-D-DCB; made in-house at Parke-Davis) was added at this point for 1 h of preincubation. The cultures were then challenged with 0.5 μ M staurosporine for up to 16 h, as described previously (19, 20).

Protein extraction and analysis. At the end of an experiment, the medium was first removed and the attached cells were washed with TBS–EDTA twice. Protein extraction was done as previously described based on cell lysis with SDS, protein precipitation with trichloroacetic acid and resolubilization with Tris base (40). Protein samples were analyzed for protein concentration with a modified Lowry assay (BioRad). Equal amounts of total protein (15 μg) were loaded to each lane and run on SDS–PAGE (4–20% acrylamide) with a Tris–glycine running buffer system and then transferred onto a PVDF membrane (0.2 μ m) with Tris-glycine buffer system using a semidry electrotransfering unit (BioRad) at 20 mA for 1.5–2 h. The blots were probed with an anti-α-spectrin (monoclonal, Chemicon) antibody or anti-calpastatin antibody (monoclonal, Chemicon), a biotinylated second antibody, and avidin conjugated with alkaline phosphatase. The blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. Densitometric analysis of Western blots was performed using a color scanner (Umax UC630) and the NIH program Image 1.5.

Calpain inhibition assay. Calpains were assayed in a microplate system with casein as substrate (31). Different amounts of cell extract or purified calpastatin preparations were added to the reaction mixture. The calpastatin concentrations that resulted in 50% calpain inhibition (IC_{50}) were determined by a curve fitting program (Sigma plot) (31).

Purification of human erythroid LMW calpastatin. Human LMW calpastatin was purified from erythrocytes. Isolated packed erythrocytes were lysed hypotonically in 20 mM Tris–HCl, 1 mM DTT, and 5 mM EGTA (pH 7.4 at 4°C) with 2 mM PMSF. Calpastatin in the hemolysate was purified by the following chromatographic steps: (i) DEAE–Sephacel chromatography (column diameter 2.5×30 cm), (ii) 5 min heating at 90°C and clarification by centrifugation (3500*g* for 10 min), (iii) omega-hexylamine–agarose column chromatography (1.5×5.5 cm), and (iv) gel filtration column chromatography (Sephacryl S-200, 1.5×60 cm). The activity was followed by assaying against μ-calpain. The purified LMW calpastatin exists in a 68-K form.

Recombinant calpastatin expression and purification. A cDNA fragment encoding the I–III domains of human calpastatin was isolated by PCR from a cDNA library prepared from total RNA isolated from SH-SY5Y neuroblastoma cells using the oligonucleotides 5′-GTA CAT ATG AAG TCA GGC ATG GAT GCT-3′ and 5′-CAG AAT TCT TAA CTC TGC GAG GTA TCA CGG G-3′. After transfer to the expression vector PT7-7 (USB, Cleveland, OH) calpastatin expression was induced with 0.4 mM isopropyl β-thiogalactoside (IPTG) for 3 h prior to harvesting and resuspending the bacteria in lysis buffer (20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM 2-mercaptoethanol). Lysates were prepared by lysozyme and DNase treatments in the presence of sodium deoxycholate and the cell free preparation was subjected to heat treatment at 100°C for 15 min. The heat stable supernatant was further purified on an immunoaffinity column containing a monclonal antibody 3.1 raised against recombinant calpastatin domain 1 (Takara Biochemicals, Japan) coupled to CNBr activated Sepharose (Pharmacia, Picsataway, NJ). The bound calpastatin was eluted with 0.1 M glycine/HCl, pH 2.5, and dialyzed against Tris-buffered saline (TBS) before use.

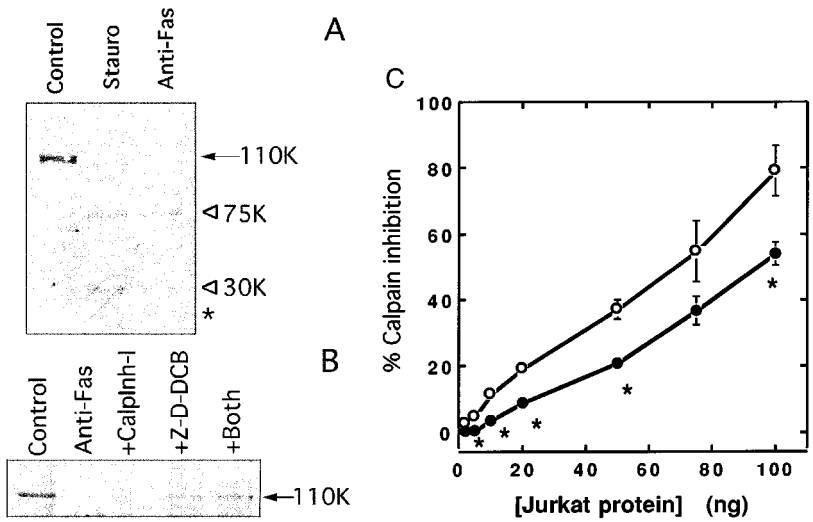

FIG. 1. HMW calpastatin degradation in Jurkat T-cell apoptosis. In A, Jurkat T-cells were either control or treated with 0.5 μM staurosporine (Stauro) or anti-Fas antibody (300 ng/ml) for 3 h. B Jurkat cells were either control or challenged with anti-Fas without or with a 1-h preincubation of 10 μM CalpInh-I or Z-D-DCB or both. HMW calpastatin fragmentation was followed by Western blot. The solid arrow represents the intact protein while the triangles indicate the major fragments. The asterisk indicated the anti-Fas band detected by the secondary antibody. (C) Total protein from control (open symbols) or anti-Fas treated Jurkat (closed symbols) was extracted with Triton X-100 and subjected to calpastatin activity measurement against μ-calpain. Data are mean ± SEM ($n = 3$). *$P < 0.05$ (Student T-test).

Digestion of purified or recombinant calpastatin by caspases. Calpastatin proteins were subjected to purified recombinant caspase-1, -2, -3, -4, -6, or -7 (32) with a substrate to protease molar ratio of 20:1 (unless stated otherwise) in 100 mM Hepes (pH 7.2 at 25°C), 10 mM dithiothreitol (DTT), 10% (v/v) glycerol, and 1 mM EGTA (for caspase) or 1 mM $CaCl_2$ (for calpain) for 90 min. The digestion was neutralized by the addition of equal volume of SDS-containing sample buffer for PAGE. Samples were divided into three sets. All were subjected to electrophoresis. One gel was stained with Coomassie blue while the other two were electrotransferred to blotting membrane. The blots were then probed with anti-calpastatin antibody.

N-terminal sequencing. Digested recombinant protein samples (100 μ g) (see above method) were arrested with SDS sample buffer. The samples were then run on 4–20% SDS–PAGE. Afterward, protein bands were transferred to PVDF membrane using 20 mM CAPS, 10% methanol (pH 11.0) for 60-75 min. The membrane was rinsed with water, stained with 0.5% Coomassie blue in 50% methanol, and detained briefly with 100% methanol. Upon drying, the bands of interest were cut out and subjected to N-terminal sequence determination using a Applied Biosystem protein sequencer.

Caspase cleavage of in vitro translated HMW and LMW calpastatin. HMW (110 K) and LMW (68 K) calpastatin cDNAs were obtained by RT-PCR on RNAs from human THP.1 and Jurkat cell lines. PCR primers were designed based on the published human calpastatin cDNA sequence (33, 34). Nucleotide sequences of the subcloned PCR fragments were confirmed. The methionine at residue 286 in human calpastatin was used to initiate translation of LMW calpastatin (amino acids 286 to 709). [^{35}S]methionine-labeled HMW and LMW calpastatins were made using a TNT-coupled transcription and translation system (Promega). Caspase cleavage reactions were performed in assay buffer containing 100 mM Hepes, pH 7.5, 20% glycerol, 0.5 mM EDTA, and 5 mM DTT at 37°C for 30 min. Cleavage reactions were done in a 20-μL volume containing 2 μL of the appropriate reticulocyte lysate plus caspase-1 (2 μg/mL), caspase-2 (25 μg/mL), caspase-3 (0.5 μg/mL), caspase-4 (2 μg/mL), caspase-5 (15 μg/mL), caspase-6 (2 μg/mL), caspase-7 (2 μg/mL), or assay buffer only. Proteolytic activity of the recombinant caspases was confirmed on appropriate peptide substrates and by cleavage of [^{35}S]methionine-labeled PARP (data not shown). Reactions were stopped by the addition of SDS sample buffer and heated at 100°C for 3 min. Reaction products were analyzed on 10–20% tricine SDS–PAGE.

RESULTS

Fragmentation of HMW Calpastatin in Early Apoptosis

Jurkat T-cells, upon challenge with anti-Fas antibody (600 ng/mL) or with staurosporine (0.5 μ M), undergo rapid apoptosis (19). Under the same conditions, we examined the integrity of calpastatin. On a Western blot, only the HMW form (apparent M_r 110 K) of calpastatin was detected in control Jurkat cells (Fig. 1A). Three hours after the induction of apoptosis by either staurosporine or anti-Fas, two major fragments with apparent M_r of 75 K and 30 K were clearly detected, accompanied by the disappearance of the intact calpastatin (Fig. 1A). Application of caspase inhibitor Z-D-DCB, but not calpain inhibitor I reduced the loss of calpastatin (Fig. 1B). The calpastatin protein from control and anti-Fas treated cells (for 3 h) were isolated by Triton X-100 extraction followed by brief boiling. When these protein extracts were assayed against μ-calpain activity (35), we found that the calpastatin activity from cells treated by anti-Fas was slightly but significantly lower than that in control cells (Fig. 1C). Measurement of cell death by LDH release showed that there was an increase in cell death at 3 h of apoptotic challenge but the LDH release became maximal only after 16–20 h (data not shown).

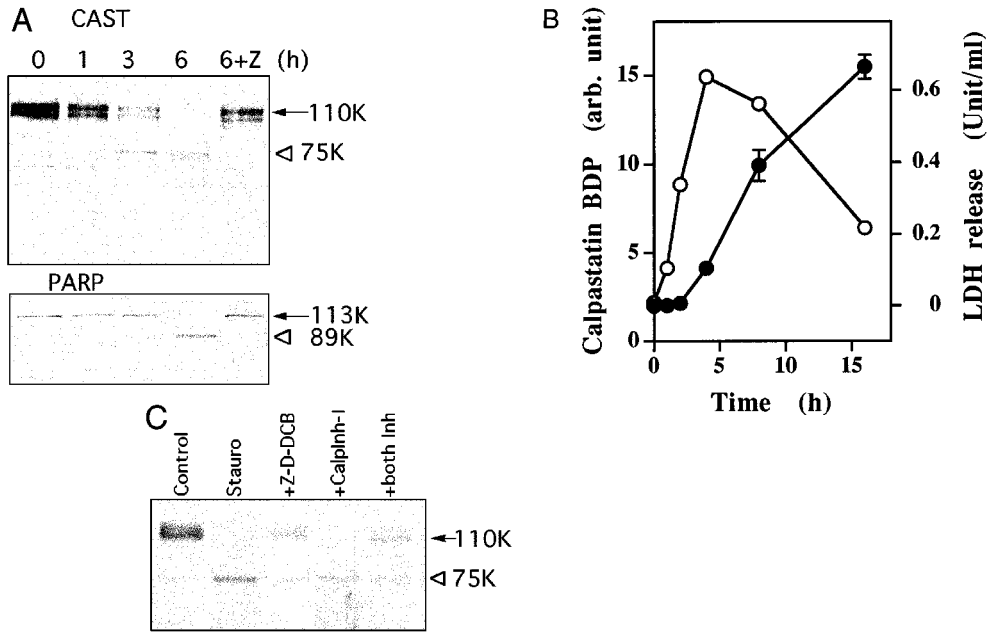

FIG. 2. Limited fragmentation of HMW calpastatin in apoptotic SY5Y neuroblastoma cells. A, SY5Y cells were subjected to 0.5 μM staurosporine challenge for 0, 1, 3, or 6 h (with or without preincubation of 50 μM Z-D-DCB). The time course of HMW calpastatin and PARP fragmentation was followed by Western blot. The solid arrows represent the intact protein while the triangles indicate the major fragment. B, The amount of 75-K calpastatin BDP (open circles) was quantified in a time course of staurosporine challenge (0.5 μM), in comparison to cell death by LDH release (solid circles). Calpastatin BDP data are generated with pooled protein extracts from four wells for each time point while LDH data are mean ± SEM ($n = 4$); *$P < 0.05$ (Student's T-test). C, SY5Y cells were either control or challenged with staurosporine for 16 h with or without preincubation of 10 μM CalpInh-I or Z-D-DCB or both.

When SY5Y cells were challenged with staurosporine, apoptosis occurs in 6–8 h (20, 36). Again, HMW calpastatin degradation occurred as early as 3 h and continued to progress. Only one major fragment of 75 K was detected in this case (Fig. 2A). PARP, a well-established caspase-3 substrate (15), was also degraded concomitantly (Fig. 2A). When the time course of 75 K calpastatin breakdown product formation was compared to cell death, it is obvious that calpastatin breakdown precedes cell death (Fig. 2B). As in the case of Jurkat cells, caspase inhibitor Z-D-DCB, but not calpain inhibitor I, provided partial protection against calpastatin loss even at 24 h (Fig. 2C).

Caspase Cleavage of In Vitro Translated HMW and LMW Calpastatin

To investigate the caspase members potentially involved in calpastatin breakdown during apoptosis, [^{35}S]methionine-labeled HMW and LMW calpastatin were generated by *in vitro* translation then digested with purified recombinant caspase-1, -2, -3, -4, -5, -6, and -7 (Figs. 3 and 4). HMW (anomalous apparent M_r 110 K) calpastatin was not significantly cleaved by caspase-2, -4, -5, and -6 (Fig. 3A). Caspase-7 (Mch-3) cleaved HMW calpastatin generating two major products that migrate on SDS–PAGE at M_r 75 K and 35 K. Caspase-3 (CPP32) appeared to cleave calpastatin at the same site as caspase-7, plus an additional site(s) within the 35 K product to yield major polypeptides of 20 and 15 K. Caspase-1 (ICE) cleaved HMW calpastatin at multiple sites, generating five major polypeptide products that migrated at M_r 75, 60, 35, 23, and 14 K (Figs. 3A and 3B). A time course of caspase-1 cleavage showed that proteolysis of HMW calpastatin was rapid with the major fragments generated within 5 min (Fig. 3B). LMW (anomalous apparent M_r 68 K) calpastatin was also degraded by caspase-1 to fragments that migrated at M_r 35, 30, 26, 20, and 13 K (Fig. 4). In contrast LMW calpastatin was not significantly cleaved by caspase-3 and -7 or by the other four caspases tested.

Digestion of Purified LMW Calpastatin by Caspase-1

To take another approach, purified erythroid LMW calpastatin (again with anomalous high M_r 68 K) was digested by caspase-1 and yielded multiple fragments (Fig. 5A). Such fragmentation was fully inhibited by caspase inhibitor Z-D-DCB. We also examined the effects of extensive fragmentation on the inhibitory activity of LMW calpastatin. When compared to the intact protein, the caspase-1 digested calpastatin exhibited slightly reduced potency toward both μ- and

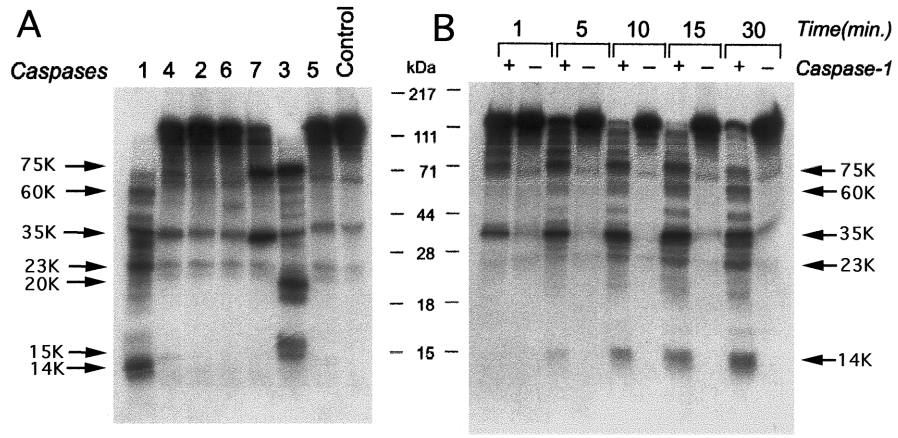

FIG. 3. Cleavage of *in vitro* translated HMW calpastatin by caspases. A, [^{35}S]methionine-labeled HMW calpastatin (110 K) was generated by *in vitro* translation and then subjected to digestion with caspase-1 (2 μg/ml), caspase-2 (25 μg/ml), caspase-3 (0.5 μg/ml), caspase-4 (2 μg/ml), caspase-5 (15 μg/ml), caspase-6 (2 μg/ml), or caspase-7 (2 μg/ml). B, *In vitro* translated HMW calpastatin (110 K) was digested with or without caspase-1 (1 μg/ml) for 1, 5, 10, 15, or 30 min. The samples were analyzed by SDS–PAGE and autoradiography. The arrows indicate the major cleavage product (75, 60, 35, 23, and 14 K for caspase-1, 75 and 35 K for caspase-7, and 75, 35, 20, and 15 K for caspase-3).

m-calpain (Figs. 5B and 5C). The concentrations that caused 50% inhibition (IC_{50}s) of μ-calpain were 7.7 ± 0.3 nM for intact LMW calpastatin and 13.4 ± 0.13 nM ($P < 0.02$) for caspase-1-digested calpastatin, respectively. Their respective IC_{50}s against m-calpain were 3.1 ± 0.6 and 6.0 ± 0.4 nM ($P < 0.02$).

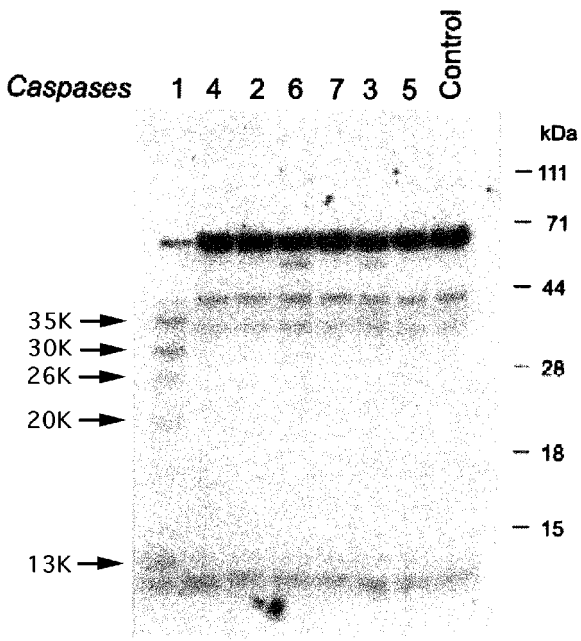

FIG. 4. Cleavage of *in vitro* translated LMW calpastatin by caspases. *In vitro* translated LMW calpastatin (68 K) was digested with caspase-1, -2, -3, -4, -5, -6, or -7 (caspase concentrations are as for HMW calpastatin digests in Fig. 3A for 30 min). Samples were then analyzed by SDS–PAGE and autoradiography. The arrows indicate the major cleavage products generated by caspase-1 (35, 30, 26, 20 and 13 K).

Identification of Caspase-1 and 3 Cleavage Sites in Recombinant Calpastatin

To elucidate the major cleavage sites in calpastatin, we took advantage of the availability of recombinant calpastatin domain 1–3 (CAST(DI–III); Met + residue 128–501). Again, it is an anomalously high M_r on SDS–PAGE (60 K) (Fig. 6A). When this purified calpastatin was subjected to digestion with caspase-1 in a 20:1 mass ratio, extensive fragmentation was observed (48, 46, 36, 35, 17, 15, and 14 K) (Fig. 6A). By contrast, under the same conditions, caspase-3 only partially degraded calpastatin to a 48 K form. The degradation of calpastatin D1–3 by caspase-1 and -3 was inhibited by the presence of 20 μM Z-D-DCB (Fig. 6A). We also observed that the caspase-1 treated CAST(DI–III) had reduced inhibitory potency against μ-calpain (from 1.62 ± 0.07 to 2.53 ± 0.03 nM; $^*P < 0.02$, Fig. 6B).

To identify the cleavage sites for caspase-1 and -3, the fragments resolved on a polyrinylidine fluoride (PVDF) membrane after Western blotting were subjected to N-terminal sequencing analysis. The data are summarized in Table I. The intact recombinant calpastatin CAST(DI–III) has an N-terminal sequence of MKSGMDAALD. The Met_1, which was introduced during cloning, was followed by the K_{128}SGMDAALD, based on the full-length human protein (33). The 46-, 35-, and 15-K fragments apparently have the intact N-terminal (Table I). The 48-K fragment produced by caspase-1 and -3 as well as the 36-K fragment produced by caspase-1 both have a N-terminal of FTXGSPTAAG (Table I). This maps the cleavage site to $LSSD_{203}$*F_{204} in the helical region C of repeat domain I (Fig. 7). The 14-K fragment had a N-terminal of LIDTLXX-PEET (Table I) and was derived from the cleavage

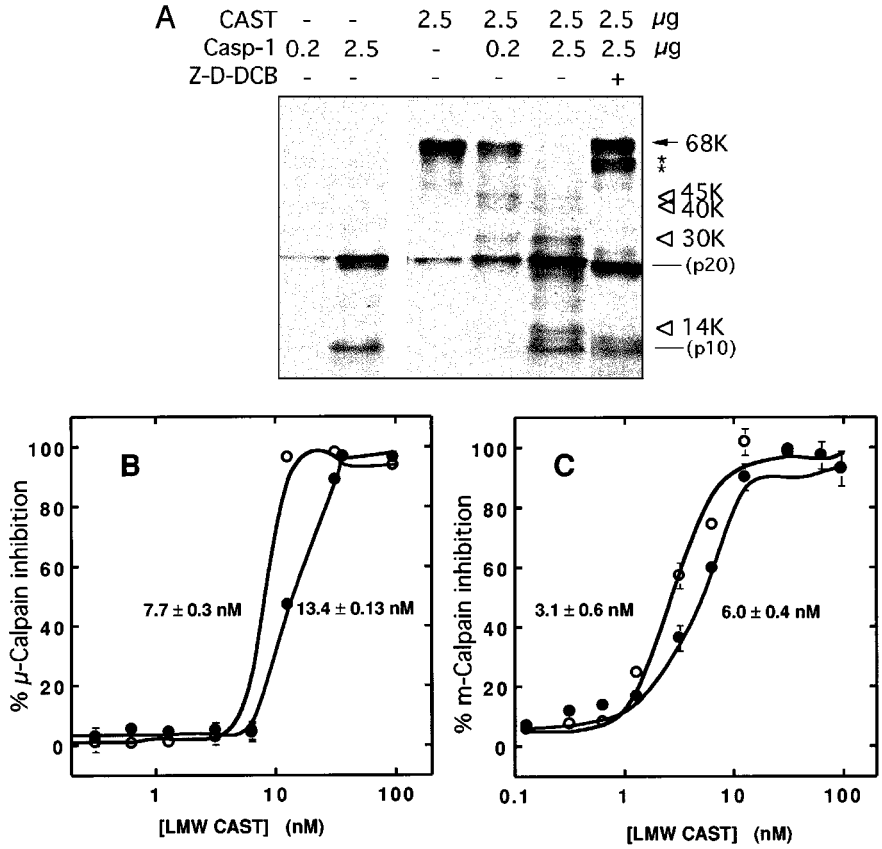

FIG. 5. Digestion of purified human erythroid LMW calpastatin by caspases. A, human LMW calpastatin (2.5 μg) was digested with various amount of caspase-1 (0, 0.2, and 2.5 μg) for 30 min. The samples were then analyzed by SDS–PAGE and Coomassie blue-staining. The solid arrow represents the intact protein while the triangles indicate the major fragments. In B and C The calpain inhibitory activity of either control (open symbols) or caspase-1-digested calpastatin (1:1 ratio) (closed symbols) (see A) were measured against μ-calpain (B) or m-calpain (C). The corresponding IC_{50} was illustrated on the graph in each case. Data shown are mean ± SEM.

$ALDD_{137}$*L_{138} in helix A in repeat I (Fig. 7). Last, a 17-K fragment had an N-terminal of SLGKKEADPE (Table I) and was derived from $ALAD_{404}$*S_{405} in helix A of domain III (Fig. 7). Each of the cleavage sites fulfilled the aspartate residue requirement in the P1 position. It should be noted that due to the anomalous migration rate of CAST(DI–III) on SDS–PAGE, the apparent M_r of some of its fragments did not necessarily match with their actual molecular weight.

DISCUSSION

In this study, we have reported for the first time that HMW calpastatin is among a growing list of proteins fragmented during apoptosis. Importantly, calpastatin breakdown precedes cell death (Fig. 2). In apoptotic Jurkat T-cells, HMW calpastatin was degraded to 75-K and 35-K immunoreactive fragments while in apoptotic SY5Y cells, the 110-K protein was degraded to a major immunoreactive fragment of 75-K (Figs. 1 and 2). In both cell types, fragmentation of HMW calpastatin during apoptosis was blocked by a caspase-specific inhibitor carbobenzoxy–Asp–$CH_2OC(O)$–2,6-dichlorobenzene (19) The difference in calpastatin fragmentation patterns in the two cell types led us to suspect that different caspases might be responsible. Indeed *in vitro* translated HMW calpastatin was degraded to different patterns by different caspases (Fig. 3): caspase-1 produced an extensive fragmentation profile, caspase-3 produced major fragments of 75, 35, 20, and 15 K, and caspase-7 produced fragments of 75 and 35 K (Fig. 3). To investigate this further, recombinant CAST(DI–III) was digested with caspase-1 and -3 (Fig. 6, Table I). We identified that helices A and C in domain I as well as helix A in domain III were attacked by caspase-1 (Table I). On the other hand, caspase-3 produced only one major cleavage in helix C of domain I (Table I). Not surprisingly, all the cleavage sites have the required Asp in the P1 position but none have the caspase-3 preferred Asp in the P4 position. It has been noted that Jurkat T-cells expressed both caspase-1 and capase-3 (37) while SY5Y cells expressed caspase-2 and -3 but not caspase-1 (19). Thus, we propose that caspase-1

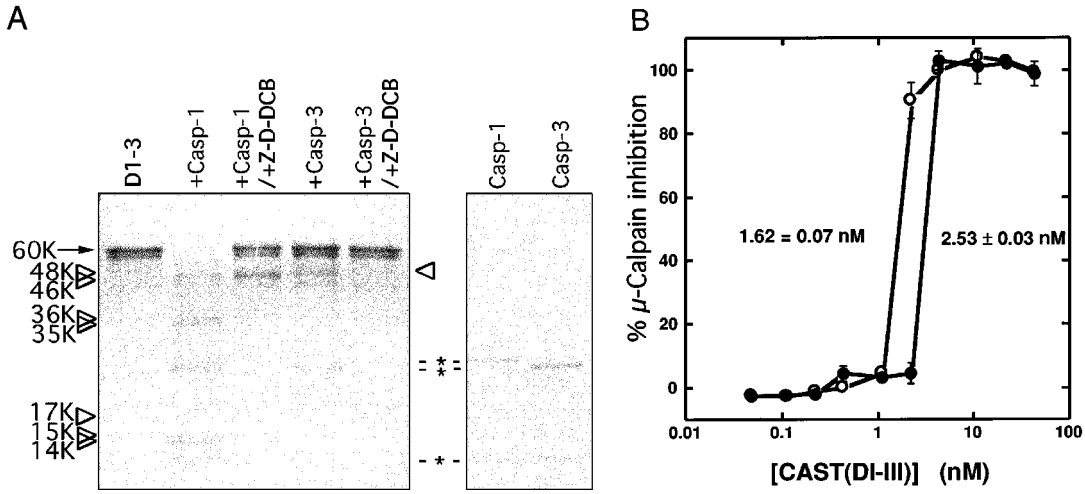

FIG. 6. Digestion of recombinant calpastatin by caspase-1 and caspase-3. (A) Human recombinant calpastatin D1–3 (3 μg) was digested with 0.2 μg/mL of caspase-1 or -3 for 60 min with or without 20 μM Z-D-DCB. The solid arrow represents the intact protein while the triangles indicate the major fragments. (B) The calpain inhibitory activity of either control (open symbols) or caspase-1-digested recombinant calpastatin (closed symbols) (see A) were measured against μ-calpain. The corresponding IC_{50} was illustrated on the graph in each case. Data shown are mean ± SE.

contributes to the fragmentation of calpastatin in Jurkat while caspase-3 and/or other caspases contribute to the limited calpastatin fragmentation in SY5Y cells.

As HMW calpastatin represents the full-length protein containing all three identified cleavages sites in domains I and III for caspase-1 and it would be expected to be cleaved into multiple fragments by caspase-1. HMW calpastatin also possesses the single caspase-3 cleavage site in domain I and would be expected to give rise to fragments of 75 and 35 K. These predictions were confirmed in the digestion study using *in vitro* translated HMW calpastatin (Fig. 3). Following the above rationale, the LMW calpastatin, which contains only domains II, III, and IV, would no longer have any caspase-3 cleavage site but still contains at least one caspase-1 site in domain III. Thus we would predict that the LMW calpastatin would be sensitive to caspase-1 but not caspase-3. Consistent with that, *in vitro* translated LMW calpastatin was not cleaved by six of seven recombinant caspases tested, the one exception being caspase-1 (Fig. 4). In fact the multiple fragmentation pattern of LMW calpastatin generated by caspase-1 suggests that additional cleavages for caspase-1 exist in domain IV, which are absent in the recombinant protein (Fig. 4, 7).

TABLE I

N-Terminal Sequence of Major CAST(DI–III) Fragments Generated by Caspase-1 and -3

Fragment	Determined N-terminal sequence	Predicted cleavage site
60 K - Intact	KSGMDAALD	(N-terminal[a] $=MK_{128}$...)
48 K[b]	FTXGSPTAAG	$LSSD_{203}$ * F_{204}
46 K	MKSGMDAXLD	(N-terminal)
36 K	FTXGSPTAAG	$LSSD_{203}$ * F_{204}
35 K	MKSXMDAXLD	(N-terminal)
17 K	SLGKKEADPE	$ALAD_{404}$ * S_{405}
15 K	MKSGMDAXLD	(N-terminal)
14 K	LIDTLXXPEET	$ALDD_{137}$ * L_{138}

[a] N-terminal of recombinant CAST(DI–III) starts with an added Met, followed by human CAST residue 128 (33).

[b] The 48 K fragment was also produced by a capase-3 cleavage between D_{203} and F_{204}.

Two groups have independently reported that in ICE (caspase-1) knockout mice stimulated with LPS, along with the expected marked decrease of IL-1β levels in the plasma, the mature IL-1α levels were also partially reduced (10, 11). It is also well established that, while ICE/caspase-1 is the pro-IL-1β processing enzyme, calpain is the preferred pro-IL-1α processing enzyme (38–40). Thus, it is possible that ICE might indirectly "activate" calpain via calpastatin degradation. In the ICE knockout, the lack of ICE would then prevent this upregulation of calpain activity. To test this hypothesis, we examined the impact of caspase-mediated proteolysis on calpain inhibitory activity of calpastatin. It is worth noting that all three identified caspase cleavage sites are located in the helical regions of their respective inhibitory unit (domain I or III) (Fig. 7). These helical regions were previously found not to interact with the catalytic domain of calpain but rather with the calcium-binding domains of calpain (24). Thus, despite extensive fragmentation of recombinant calpastatin D1–3 and erythroid LMW calpastatin by caspase-1, only a less than twofold drop in inhibitory activity was observed in each case

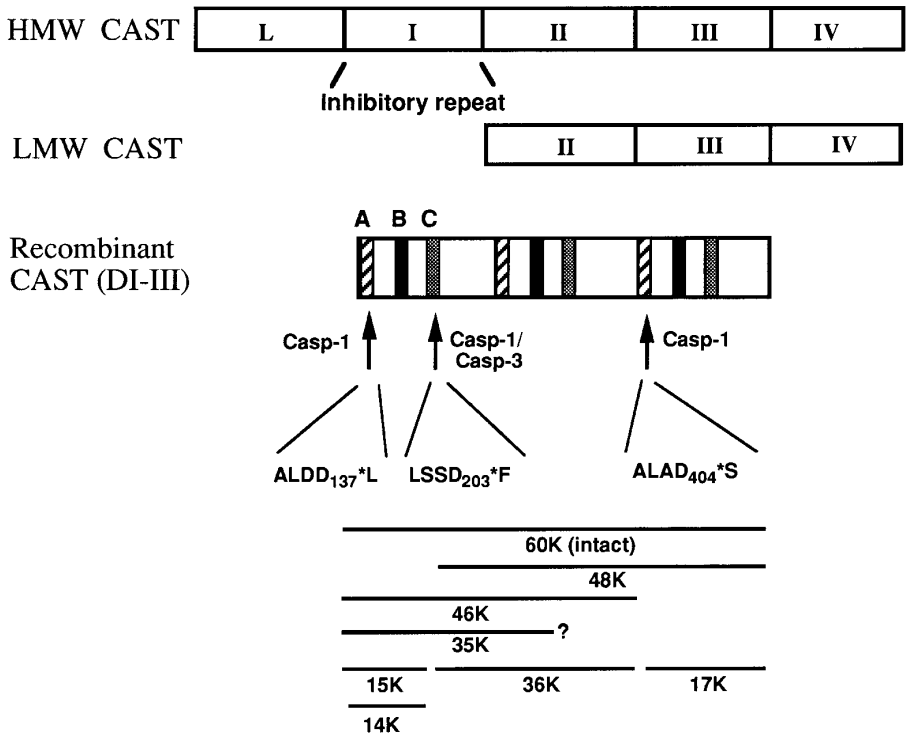

FIG. 7. Schematic of calpastatin fragmentation by caspase-1 and -3. Schematic model of HMW calpastatin (CAST) illustrates that it contains an N-terminal domain L and four homologous inhibitory repeats. On the other hand, LMW contains only repeats II to IV. Recombinant CAST contains repeats I-III only. In each repeat, conserved helical regions A (crossed) and C (shaded) flank the conserved inhibitory core region B (solid). The identified single caspase-3 cleavage site as well as two additional caspase-1 sites are illustrated. The bottom of the diagram shows possible origins of the major fragments.

(Figs. 5C, 5D, 6B). Thus, it seems unlikely that a modest drop of calpastatin activity during apoptosis is sufficient to alter the overall calpain activity levels *in vivo* (Fig. 1C). We thus propose that calpastatin fragmentation might have yet unidentified functions to facilitate the cross-talk between caspases and calpains during apoptosis. It is also possible that calpastatin fragmentation might have other functions outside of calpain activation in apoptosis. Future studies employing cells or animals expressing caspase-resistant calpastatin mutant would shed light on the role of calpastatin fragmentation during apoptosis.

Recently, calpastatin has been identified as an antigen that stimulates antibody production in patients who suffer from autoimmune disorders (41, 42). This is reminiscent of several other caspase substrates that also are autoantigens (DNA-dependent kinase, PARP) U1-70 kDa and α-spectrin (16). These autoantigenic protein fragments are likely generated during physiological apoptosis. Thus calpastatin joins the growing number of caspase substrates that also serve as autoantigens.

Calpastatin fragmentation was previously reported by Pontremoli *et al.* (29) under *in vitro* conditions. The authors proposed that when calpain is in excess, calpastatin is susceptible to calpain digestion. Mohan and Nixon (43) also reported that HMW calpastatin was processed into 71, 41, and 31 K fragments in Y-79 retinoblastoma cells exposed to 2–4 mM calcium. The calpastatin processing was inhibited by calpain inhibitors. By contrast, we demonstrated in this study that calpastatin was degraded not by calpain but by caspases during apoptosis. We show that a caspase inhibitor could reduce calpastatin breakdown in cultured cells while a calpain inhibitor was ineffective (Figs. 1 and 2). It is possible that calpastatin can be alternately susceptible to calpain and caspase proteolysis under different conditions. For example, in rat cerebellar granule neurons cells, α-spectrin is degraded by calpain alone in maitotoxin-induced necrosis but by both calpain and caspase in potassium deprivation-induced apoptosis (19). By contrast, α-spectrin was cleaved only by caspase in anti-Fas-induced apoptosis in Jurkat T-cells (19, 44).

A previous study reported that Alzheimer's disease (AD) brains have lower levels of calpastatin activity (45). Recently, Nixon and colleagues also found a significant loss of calpastatin protein in the AD brains (46). Saido *et al.* (47) recently showed that calpastatin was proteolyzed in ischemic rat brains subjected to ischemia. It would be premature at this point to sug-

gest that such calpastatin loss was due to caspase activation. Further work will be needed to investigate this possibility.

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