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Cleavage of the calpain inhibitor, calpastatin, during apoptosis

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Abstract

Calpain activity is thought to be essential for the execution of apoptotic cell death in certain experimental models. In the present study, the physiological inhibitor of calpain, calpastatin, was found to be cleaved in three different apoptotic systems. The 110–120 kDa calpastatin protein of Jurkat T-lymphocytes and U937 monocytic leukemia cells was cleaved to a 65–70 kDa form after the induction of apoptosis with anti-CD95 monoclonal antibody, staurosporine or TNF. Cleavage of calpastatin in apoptotic cells occurred simultaneously with the cleavage of the DNA repair enzyme, poly(ADP-ribose) polymerase. The caspase inhibitors VAD-cmk and IETD-fmk prevented calpastatin cleavage in all three systems. Calpain inhibitor I, however, suppressed calpastatin cleavage only during TNF-induced apoptosis. Other protease inhibitors, such as lactacystin and pepstatin A, did not confer any significant protection against apoptotic calpastatin cleavage. The results from *in vitro* incubations with cell lysates and purified enzymes showed that calpain I, calpain II and recombinant caspase-3, all cleaved calpastatin, with varying efficiency. In conclusion, the results of the present study suggest that caspases may cleave calpastatin and thus, regulate calpain activity during apoptotic cell death.

Keywords: apoptosis calpastatin; calpain; caspase

Abbreviations: PBS, phosphate-buffered saline; PMSF, phenylmethyl sulfonyl fluoride; TNF, tumor necrosis factor-α; VAD-cmk, Val-Ala-Asp-chloromethylketone; IETD-fmk, Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl-ketone; mAb, monoclonal antibody; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase

Introduction

Apoptosis is known to involve specific cleavage of certain cellular proteins, such as the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) (Nicholson *et al*, 1995) and the cytoskeletal protein α-fodrin (Martin *et al*, 1995; Vanags *et al*, 1996). Caspases (aspartic acid-specific cysteine proteases) have been recognized as the main group of enzymes responsible for apoptotic proteolysis (Zhivotovsky *et al*, 1997; Cohen, 1997). Cells undergoing apoptosis following the stimulation of death receptors, execute the apoptotic programme by activating a hierarchy of caspases, whereby the type III caspases (caspases 6,8,9 and 10) are thought to sequentially activate other caspases (Fraser and Evan, 1996; Thornberry *et al*, 1997). It is known that PARP, α-fodrin and protein kinase Cδ, among other proteins, are substrates for caspases (Nicholson *et al*, 1995; Nath *et al*, 1996; Cryns *et al*, 1996; Emoto *et al*, 1995).

Calpain (calcium-activated neutral protease) is a Ca^{2+}-dependent cysteine protease which has been implicated in proteolysis of a number of proteins not only under normal conditions, but also during apoptosis. In a number of different models, such as TNF-induced apoptosis of U937 cells (Vanags *et al*, 1996), glucocorticoid- and irradiation-induced apoptosis in thymocytes (Squier *et al*, 1994; Squier and Cohen 1997), as well as activation-induced cell death in T-lymphocytes (Sarin *et al*, 1994), calpain appears to play a key function. Some overlap seems to exist between the substrate specificities of caspases and calpains, since the long list of known calpain substrates includes α-fodrin (Croall and DeMartino, 1986; Martin *et al*, 1995; Vanags *et al*, 1996) and protein kinase C (Inoue *et al*, 1977). However, the pattern of the substrate cleavage is different between the two families.

Calpain constitutes a large family of distinct isozymes differing in structure and distribution (Saido *et al*, 1994). The two ubiquitous family members are μ-calpain and m-calpain (or calpain I and II, respectively). A specific endogenous protein inhibitor, calpastatin, modulates calpain activity *in vivo* (DeMartino and Croall, 1984). Calpastatins, which vary in size from 70–140 kDa depending on the tissue type, are derived from a single calpastatin gene by differential mRNA splicing (Lee *et al*, 1992) and post-translational modification by phosphorylation (Adachi *et al*, 1991) and proteolysis (Nakamura *et al*, 1989). Calpastatin functions as an inhibitor with high affinity and strict specificity for calpain. It has been reported to act as a suicide substrate when binding to calpain (Nagao *et al*, 1994). Thus, the ratio of calpastatin to calpain is an important factor affecting the extent of calpain activity within cells. Calpastatin contains four functional repeats of about 140 amino acids each, that are homologous to one another (Maki *et al*, 1987; Emori *et al*, 1988). Within each repeat there are three highly conserved clusters, referred to as the central consensus sequence, the N-terminal and C-terminal flanking sequences. Numerous studies have demonstrated the inhibitory activity of peptides based on the central consensus sequence and shown this sequence to be both necessary and sufficient for specific inhibition of calpain (Croall and McGrody, 1994).

The present study demonstrates that in U937 monocytic cells and Jurkat T-lymphocytes calpastatin is cleaved upon induction of apoptosis by TNF, anti-CD95 monoclonal antibody (mAb), or staurosporine. The effects of protease inhibitors suggest that the main enzyme responsible for calpastatin cleavage during apoptosis is a caspase.

Results

The effects of protease inhibitors on calpastatin cleavage during CD95-mediated apoptosis

In Jurkat T-lymphocytes intact calpastatin is about 120 kDa in size, as determined by SDS polyacrylamide gel electrophoresis and Western blotting (Figures 1a and Figure 2a). The formation of a ~70 kDa breakdown product of calpastatin and the cleavage of PARP were observed to occur concomitantly, upon the induction of apoptosis with anti-CD95 mAb (250 ng/ml) (Figure 1). After 4 h treatment, significant amounts of calpastatin and PARP were cleaved.

In these cells apoptotic-associated DNA damage was dramatically reduced after pre-treatment with the broad-range caspase inhibitor VAD-cmk (Table 1). Similarly, cleavage of both calpastatin and PARP were significantly blocked after pre-treatment with VAD-cmk (Figure 1). IETD-fmk (20 μM), an inhibitor of the type III caspases, also prevented anti-CD95-induced calpastatin cleavage in Jurkat cells (data not shown). The calpain inhibitor I and the proteasome complex inhibitor, lactacystin, had no detectable effects on the proteolysis of calpastatin in these cells (Figure 1).

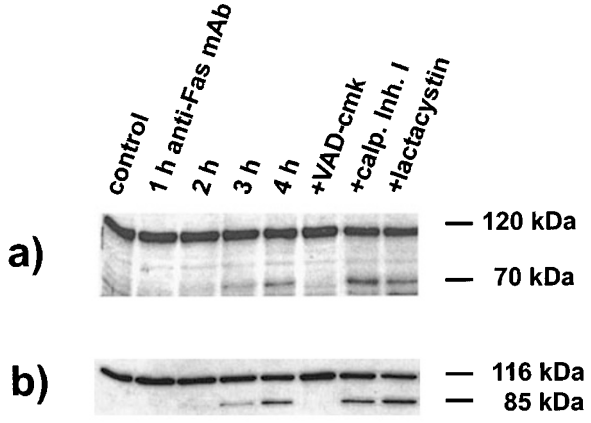

Figure 1 CD95-mediated calpastatin proteolysis and apoptosis in Jurkat cells. Jurkat cells were treated with anti-CD95 mAb (250 ng/ml) for up to 4 h in the presence (4 h) or absence of protease inhibitors, 20 μM VAD-cmk, 50 μg/ml calpain inhibitor I, or 10 μM lactacystin with 30 min pre-incubation. The incubation was stopped by addition of cold PBS and centrifugation of samples. Samples were then prepared for Western blot or FACS analysis as described in Materials and Methods. (**a**) The kinetics of calpastatin cleavage after stimulation with anti-CD95 mAb and the effect of protease inhibitors. The blot is representative of five similar experiments (**b**) The kinetics of PARP cleavage in CD95-mediated apoptosis and the effects of protease inhibitors. The membrane shown in (**a**) was stripped and re-probed with anti-PARP mAb. The blot is representative of three similar experiments

Apoptosis and proteolysis of calpastatin in staurosporine-treated Jurkat cells

Treatment of Jurkat cells with staurosporine (5 μM) induced the cleavage of both calpastatin and PARP. This occurred within 2 h, and by 4 h the majority of the intact calpastatin was converted to the ~70 kDa breakdown product (Figure 2). The caspase inhibitor, VAD-cmk (20 μm), partially inhibited staurosporine-induced calpastatin cleavage (Figure 2a). As in CD95-treated cells, the cleavage of calpastatin was not inhibited by calpain inhibitor I or lactacystin. Nevertheless, all the inhibitors tested here, conferred some degree of protection against staurosporine-induced DNA damage as measured by flow cytometry (Table 1).

Effects of protease inhibitors on TNF-induced calpastatin cleavage and apoptosis of U937 cells

In monocytic U937 cells, the intact calpastatin band had an apparent molecular weight of 110 kDa (Figure 3a), i.e., it seemed somewhat smaller than the calpastatin of Jurkat cells. In addition to the 110 kDa band, another immunoreactive band of ~70 kDa was detectable by the calpastatin mAb in the untreated cells. Treatment of U937 cells with TNF (30 ng/ml) induced apoptosis and the appearance of a ~65 kDa cleavage product of calpastatin. The cleavage product was detectable within 2 h, concomitant with a decrease in the density of both the 110 kDa and the 70 kDa bands (Figure 3a). The cleavage of PARP followed a similar time course (Figure 3b).

Table 1 The effects of various protease inhibitors on DNA breakdown during apoptosis

Inhibitor	% Apoptosis		
	Jurkat cells+anti-CD95	Jurkat cells+ staurosporine	U937 cells+TNF
	31.5	32.8	48.2
VAD-cmk (20 μM)	7.2	23.4	3.9
Calp. Inh (50 μg/ml)	29.7	24.1	26.2
Lactacystin (10 μM)	26.0	25.3	41.0
Pepstatin A (100 μM)	N.D.	N.D.	41.0

Cells were treated with the inhibitors prior to induction of apoptosis as in Figure 1. Samples were analyzed using a flow cytometer and the percentage apoptosis was calculated as described in Materials and Methods. The background level of DNA in the pre-G1 peak was 5.7% and 4.4% in Jurkat and U937 cells, respectively. The data presented here is the representation of a typical experiment

Prior treatment with VAD-cmk had a moderate inhibitory effect on the TNF-induced cleavage of both calpastatin and PARP, however, it effectively prevented accumulation of the pre-G1 apoptotic DNA peak (Figure 3 and Table 1). IETD-fmk (20 μM) also inhibited TNF-induced calpastatin cleavage (data not shown). As reported previously (Vanags *et al*, 1996), calpain inhibitor I suppressed TNF-induced apoptosis in U937 cells. As depicted in Figure 3, calpain inhibitor I was also an effective inhibitor of TNF-induced cleavage of both calpastatin and PARP. Lactacystin only slightly inhibited calpastatin and PARP cleavage (Figure 3b), but was not nearly as effective as calpain inhibitor I at protecting against DNA damage (Table 1). Cathepsin D has recently been implicated in TNF-induced apoptosis (Deiss *et al*, 1996), which prompted us to include pepstatin A in the panel of inhibitors during the studies using TNF. However, the cathepsin D inhibitor did not show a clear protective effect on TNF-induced apoptosis in our system (Table 1).

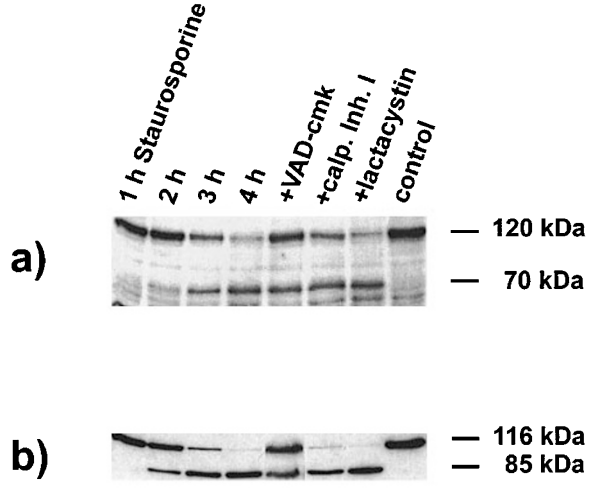

Figure 2 Staurosporine-induced apoptosis and proteolysis of calpastatin in Jurkat cells. Jurkat cells were treated with staurosporine (5 μM) for up to 4 h in the presence (4 h) or absence of protease inhibitors, 20 μM VAD-cmk, 50 μg/ml calpain inhibitor I, or 10 μM lactacystin with 30 min pre-incubation. (**a**) The kinetics of calpastatin cleavage after staurosporine treatment and the effects of protease inhibitors. The blot is representative of four similar experiments (**b**) The kinetics of PARP cleavage in stauroporine-induced apoptosis. The membrane from (**a**) was stripped and re-probed with anti-PARP mAb. The blot is representative of three similar experiments

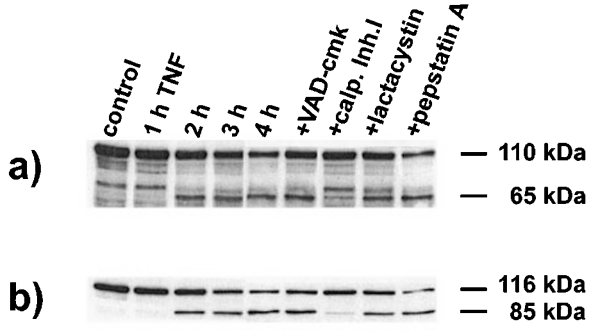

Figure 3 TNF-induced apoptosis and proteolysis of calpastatin in U937 cells. U937 cells were treated with TNF (30 ng/ml) for up to 4 h in the presence (4 h) or absence of protease inhibitors, 20 μM VAD-cmk, 50 μg/ml calpain inhibitor I, 10 μM lactacystin or 100 μM pepstatin A with 30 min pre-incubation. Cycloheximide (1 μg/ml) was added to all samples to enhance the effect of TNF. (**a**) The kinetics of calpastatin cleavage and effects of protease inhibitors. The blot is representative of five similar experiments. (**b**) The kinetics of PARP cleavage and the effects of protease inhibitors. The membrane from (**a**) was stripped and re-probed with anti-PARP mAb. The blot is representative of three similar experiments

In vitro proteolysis of calpastatin

The cleavage of calpastatin in apoptosis was prevented by both caspase inhibitors and calpain inhibitor I, depending on the model of apoptosis studied. Thus, we have two likely candidates for the enzyme responsible for apoptotic calpastatin cleavage: a caspase and/or calpain. Calpastatin has previously been reported to act as a 'suicide substrate' of calpain (Nagao *et al*, 1994). Upon incubation of Jurkat cell lysates with purified calpain I (Figure 4a), and calpain II (data not shown) we observed formation of a calpastatin breakdown product of $\sim$70 kDa. Addition of active caspase-3 to cell lysates also caused cleavage of calpastatin, as well as cleavage of α-fodrin (Figure 4).

Discussion

In recent years, proteolysis has emerged as an important feature of apoptosis. A number of different proteins are known to be cleaved during the apoptotic process, including PARP, nuclear lamins and cytoskeletal α-fodrin (Lazebnik *et al*, 1995; Zhivotovsky *et al*, 1995). In the present study we demonstrate that cleavage of the calpain inhibitory protein, calpastatin, occurs in several different models of apoptosis. Nagao and co-workers (1994) previously reported that ionomycin induces cleavage of calpastatin by calpain in epidermoid carcinoma

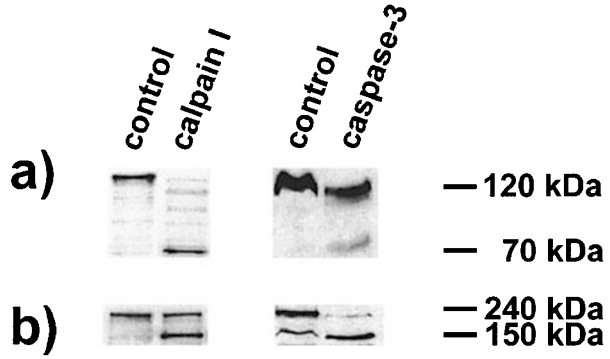

Figure 4 *In vitro* proteolysis of calpastatin. The cells were lysed as described under Materials and Methods, and treated with calpain I or II (80 ng/μg cellular protein extract, 1.5 h), or caspase-3 (1 U/μg cellular protein extract, 2 h). The enzyme digestion was stopped by addition of PAGE buffer and samples were analyzed with PAGE and Western blotting as described under Materials and Methods. (**a**) Calpastatin content of lysates after calpain or caspase treatment. (**b**) Cleavage of α-fodrin (240 kDa)

cells, with a main cleavage product of 68 kDa. The relevance of calpastatin cleavage is not at present clear. Because calpastatin can be cleaved as it inhibits calpain, depletion of calpastatin may be the result of elevated calpain activity. In TNF-induced apoptosis of U937 cells, calpastatin cleavage, as well as other features of cell death, were inhibited by calpain inhibitor I (Figure 3 and Table 1), which supports this hypothesis. Analysis of brain cortical layers from patients with Alzheimer's disease have revealed significantly decreased levels of calpastatin as compared to the brains of control subjects (Nixon *et al*, 1994), suggesting that depletion of calpastatin may be one critical factor in determining if a given level of calpain activation will lead to cellular degeneration. Calpains have also been isolated from the mitochodrial matrix of cells (Tavares and Duque-Magalhaes, 1991), which may be of potential interest in the light of recent evidence for a role of mitochondria in apoptosis (Mignotte and Vayssiere, 1998).

The results obtained using various protease inhibitors in the present study suggest that a caspase may be the main protease responsible for cleaving calpastatin during CD95- and staurosporine-mediated apoptosis. The conserved regions within the calpastatin molecule are quite rich in aspartic acid residues (Maki *et al*, 1990), with typical cleavage motifs for caspases, making it a potentially good substrate for this family of proteases (Nicholson, 1996; Thornberry *et al*, 1997). Interestingly the cleavage of the intact calpastatin at $DAID_{234}A$ or $LSSD_{238}F$, is capable of producing a fragment of $\sim$70 kDa. Although, neither of these possible cleavage sites fall within the motifs for binding to and inhibiting calpain, it is unclear whether cleavage at these sites would impair the calpain inhibitory effect of calpastatin. However, if that is the case, then one would expect to observe elevated calpain activity during the later stages of apoptosis. The specific cleavage site on calpastatin needs to be determined before any of the physiological implications of this event could be addressed.

The incubation of cell lysates with purified caspase-3, inefficiently cleaved calpastatin even though α-fodrin was almost completely cleaved by the protease. This suggests that calpastatin may not be the favoured substrate for capase-3 and that other caspases may preferentially cleave this protein. Since VAD-cmk and IETD-fmk conferred significant protection from calpastatin cleavage, it seems reasonable to speculate that a type I or III caspase could be responsible for the calpastatin cleavage during apoptosis.

Apart from calpains and caspases, calpastatin also appears to be a substrate from proteosomal activity (Mellgren, 1997). In experiments using peptide aldehydes *in vivo*, it is difficult to identify which protease - calpain or proteasome - is inhibited and involved in cellular functions, because peptide aldehydes may inhibit both calpain and proteasome activity (Mellgren, 1997). Proteasomes may also be involved in certain types of apoptosis (Sadoul *et al*, 1996). However, the most specific inhibitor available for proteasomal activity, lactacystin, did not show any significant protective effects on any of the apoptotic events studied.

In conclusion, although calpains may be involved in calpastatin cleavage in some cell types, caspases appear to be the main players in this event during apoptosis. Determining the physiological implication of calpastatin cleavage may provide the missing link between the two major classes of proteases in apoptosis.

Materials and Methods

Materials

Anti-human CD95 mAb (IgM CH-11 clone) was purchased from Medical and Biological Laboratories Co, Ltd. (Nagoya, Japan). TNF (human) was a kind gift from Dr. Grace Wong, Genentech Inc. (San Francisco, CA, USA). Anti-calpastatin mAb was from Chemicon (Temecula, CA, USA) and anti-PARP mAb was from Biomol (Plymouth Meeting, PA, USA). Anti-α-fodrin mAb was from Affiniti. Calpain inhibitor I was obtained from Boehringer Mannheim. VAD-cmk and IETD-fmk were purchased from Enzyme Systems Products (Livermore, CA, USA). Purified calpain I (porcine erythrocytes), calpain II (porcine kidney) and lactacystin were from Calbiochem. Staurosporine and propidium iodide were obtained from Sigma. Purified recombinant caspase-3 was a kind gift from Dr D W Nicholson, Merck Frosst Center for Therapeutic Research (Montreal, Quebec, Canada).

Cell culture

Jurkat cells (human leukemic T-lymphocytes) and human myeloid leukemic U937 cells were from the European Collection of Cell Cultures (London, UK). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were maintained in a logarithmic growth phase by routine passage every 2–3 days.

In vitro digestion of calpastatin

Protein extracts were prepared from either untreated Jurkat or U937 cells by resuspending the pellet in lysis buffer (20 mM Tris-HC1 (pH 7.4), 150 mM NaC1, 1 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA, 0.5% (w/v) Triton X-100, and 1 mM PMSF). The suspension was kept on ice with occasional mixing for 45 min and subsequently centrifuged at 15 000 $\times$ *g*. The supernatant was mixed with an equal volume of glycerol and stored at -70°C. Approximately 50 μg of protein was incubated with 4 μg of purified calpain I or calpain II in the presence of 0.5 mM or 5 mM $CaC1_2$, respectively, for 90 min at 30°C. For treatment with caspase-3, cell lysates were incubated with 0.33–0.66 U of purified enzyme/μg lysate protein in a buffer containing 100 mM HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, 0.1 mM EDTA, and 1 mM EGTA for 2 h at 30°C. The reactions were terminated by the addition of SDS-containing sample buffer for PAGE. Samples were then subjected to electrophoresis and electrotransfer, and the blots were probed with monoclonal antibodies against calpastatin or α-fodrin.

Preparation of cell lysates for Western blotting

Cells were washed with ice-cold PBS, pH 7.2, containing 100 μM PMSF and pelleted at 200 $\times$ *g*. Cell pellets were resuspended in lysis buffer for PAGE (62.5 mM Tris-HC1, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 1 mM PMSF) 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 120 V in 7.5% 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 NaC1, 0.05% Tween-20) containing 4% bovine serum albumin and then incubated for 1 h with anti-calpastatin mAb diluted 1 : 1000, anti-PARP mAb diluted 1 : 3000, or anti-α-fodrin mAb diluted 1 : 1000 in high salt buffer. After washing the blots four 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.).

Assessment of apoptosis by FACS analysis

The DNA content of cells was analyzed with propidium iodide staining of cells (McGahon *et al*, 1995). After appropriate treatments, cells (5×10^5 per sample) were spun down and rinsed with cold PBS. The cells were then fixed in ice-cold 70% ethanol for 20 – 30 min. After centrifugation, the pellets were resuspended in 50 μg/ml of propidium iodide containing 0.5 mg/ml of RNase A and incubated on ice for 30 min. Samples were analyzed with a Becton Dickinson FACScan flow cytometer with a 15 mW 488 nm argon laser; 10 000 cells per sample were counted. The percentage apoptosis was calculated as the area under the pre-G1 peak.

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