Non-erythroid α-spectrin breakdown by calpain and interleukin 1β-converting-enzyme-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis

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The cytoskeletal protein non-erythroid α-spectrin is well documented as an endogenous calpain substrate, especially under pathophysiological conditions. In cell necrosis (e.g. maitotoxin-treated neuroblastoma SH-SY5Y cells), α-spectrin breakdown products (SBDPs) of 150 kDa and 145 kDa were produced by cellular calpains. In contrast, in neuronal cells undergoing apoptosis (cerebellar granule neurons subjected to low potassium and SH-SY5Y cells treated with staurosporine), an additional SBDP of 120 kDa was also observed. The formation of the 120 kDa SBDP was insensitive to calpain inhibitors but was completely blocked by an interleukin 1β-converting-enzyme (ICE)-like protease inhibitor, Z-Asp-CH_2OC(O)-2,6-dichlorobenzene. Autolytic activation of both calpain and the ICE homologue CPP32 was also observed in apoptotic cells. α-Spectrin can also be cleaved *in vitro* by purified calpains to produce the SBDP doublet of 150/145 kDa and by ICE and ICE homologues [ICH-1, ICH-2 and CPP32(β)] to produce a 150 kDa SBDP. In addition, CPP32 and ICE also produced a 120 kDa SBDP. Furthermore inhibition of either ICE-like protease(s) or calpain protects both granule neurons and SH-SY5Y cells against apoptosis. Our results suggest that both protease families participate in the expression of neuronal apoptosis.

INTRODUCTION

Apoptosis is a form of cell death with the following associated characteristics: condensation of the nucleus, fragmentation of chromatin at nucleosome linkage sites, membrane blebbing and the formation of apoptotic bodies [1–3]. Apoptosis can either (1) occur physiologically at many developmental (e.g. morphogenesis by cell selection) or cell maturation (T-cell clonal selection) check points, or (2) be triggered by some form of external challenge (e.g. exposure to chemical toxins or adverse conditions such as hypoxia or withdrawal of certain growth factors) [1–3]. The identification of sequence homology between one of the *Caenorhabditis elegans* cell-death genes, *ced*-3, and the mammalian IL 1β-converting enzyme (ICE) initiated an uprise of research activities that led to both the discovery of more than 10 ICE-like cysteine proteases and their potential roles in apoptosis [4–11]. For example, overexpression of ICE in fibroblasts [12] can lead to apoptosis. A study by Nicholson et al. [13] provided strong evidence that an ICE homologue CPP32 (also called apopain) might be more relevant in the induction of mammalian apoptosis. In contrast, the participation of other families of proteases in apoptosis has been reported. For example, Chow et al. [14] and Schlegel et al. [15] have suggested that serine protease(s) may be involved because 7-amino-1-chloro-3-L-tosylamidoheptan-2-one can inhibit apoptosis in T-cells. Calpain is a calcium-activated cysteine protease that is overactivated in a number of pathological conditions normally associated with necrosis [16–18]. However, several studies have indicated that calpain is also activated in apoptosis in several haematopoietic cells [19–21].

So far, a few proteins have been found to be cleaved during apoptosis. These include poly(ADP-ribose) polymerase (PARP) [22] and a small U1 nuclear ribonucleoprotein [23]. Both PARP and the ribonucleoprotein were subsequently identified as substrates for ICE and/or its homologues (ICH; also called caspase) [13,24–26]. Non-erythroid α-spectrin (also called α-fodrin) is a calmodulin-binding protein recently found to be cleaved in apoptotic T-cells by Martin et al. [27]. The authors suggested that calpain was the protease solely responsible.

Here we extend the investigation of proteases in neuronal apoptosis because (1) apoptosis has been implicated in a number of neurodegenerative diseases such as cerebral ischaemia and Alzheimer's disease [28–30], and (2) cellular mechanisms leading to neuronal apoptosis are poorly understood [2,31]. For the first time we present evidence that α-spectrin is cleaved by both calpain and ICE-like protease(s) into distinct fragments during apoptosis of neuronal cells. We also have evidence that inhibition of calpain or ICE-like protease(s) can reduce neuronal apoptosis *in situ*.

MATERIALS AND METHODS

Rat cerebellar granule neuron cultures

Cerebellar granule neurons were isolated from 7-day-old rat pups. The meninges were removed from the cerebellum and the

Abbreviations used: CalpInh-I, calpain inhibitor I (Ac-Leu-Leu-Nle-H); ICE, interleukin 1β-converting enzyme; ICH, ICE homologue; MCA, 4-methylcoumarinamine; MTX, maitotoxin; PD150606, 3-(4-iodophenyl)-2-mercapto-(*Z*)-2-propenoic acid; MEM, minimum essential medium; NGF, nerve growth factor; PARP, poly(ADP-ribose) polymerase; PEST, protein sequence enriched with proline, glutamate/aspartate, serine and threonine residues; SBDP, α-spectrin breakdown product; s-calpain, small subunit of calpain; Z-D-DCB, carbobenzoxy-Asp-CH_2OC(O)-2,6-dichlorobenzene; Z-VAD(OEt)-DCB, carbobenzoxy-Val-Ala-Asp(OEt)-CH_2OC(O)-2,6-dichlorobenzene.

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minced tissue was treated with trypsin (0.25 mg/ml) for 15 min at 37 °C. The trypsin-treated tissue was then washed in 0.4 mg/ml DNase I for 5 min at 1000 rev./min. The tissue was triturated three times with a sterile Pasteur pipette in 0.4 mg/ml DNase I (the supernatant contained the dissociated cells). The combined supernatant was divided into four centrifuge tubes and 3 ml of 4% (w/v) BSA/1 mM $MgSO_4$ was layered underneath the supernatant. The cells were centrifuged at 2000 ***g*** for 5 min at 4 °C and washed in HBSS (pH 7.3, 20 mM Hepes, 0.59 mM EDTA) for 5 min at 1000 rev./min. The pellet was resuspended and cultured (about 1 cerebellum per 12-well plate) in 2 ml of feeding media [Dulbecco's modified Eagle's medium plus 30 mM KCl, 10% (v/v) fetal bovine serum, 5 mg/ml insulin, 100 i.u./ml penicillin and 100 μg/ml streptomycin] in a 37 °C humidified 5% CO_2 incubator. After 24 h, half of the culture medium was replaced with fresh medium containing 20 μM cytosine arabinoside. The cells remained in culture for 7 days *in vitro* before use. At the beginning of an experiment, 7-day-old cultures were pretreated with calpain inhibitors [acetyl-Leu-Leu-Nle-H (calpain inhibitor I; CalpInh-I) or 3-(4-iodophenyl)-2-mercapto-(*Z*)-2-propenoic acid (PD150606)] [32] or the ICH inhibitors carbobenzoxy-Asp-CH_2OC(O)-2,6-dichlorobenzene (Z-D-DCB), carbobenzoxy-Val-Ala-Asp(OEt)-CH_2OC(O)-2,6-dichlorobenzene [Z-VAD(OEt)-DCB] (both made in-house at Parke-Davis), Ac-DEVD-H or Ac-YVAD-H [13,33] for 2 h. The cultures were washed three times with serum-free minimum essential medium (MEM) containing only 5 mM KCl, and maintained in the same medium for 1–24 h. Unless otherwise stated the cultures were maintained in low-K^+ medium for 16 h, at which time cell viability was monitored or protein or DNA extraction was performed.

Maitotoxin (MTX) and staurosporine treatment of SH-SY5Y cells

Human neuroblastoma SH-SY5Y cells were grown on 12-well plates to confluency (about 2×10^6 per well) with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml Fungizone (amphotericin B). Cultures were washed three times with serum-free MEM. CalpInh-I (Calbiochem), PD150606 or Z-D-DCB was added at this point for 1 h of preincubation. The cultures were then challenged with the calcium channel activator MTX (0.1 nM) as described previously [34] or staurosporine (0.5 μM) for 1 h [35]. Unless otherwise stated the cultures were washed and returned to serum-free medium for 24 h, when cell viability was monitored or protein or DNA extraction was performed.

Nerve growth factor withdrawal-dependent apoptosis in PC-12

Rat phaeochromocytoma PC-12 cells maintained in MEM medium on 100 mm plates (Nunc) were differentiated by 100 ng/ml of nerve growth factor (NGF; full-length; Sigma) to sympathetic neuron-like phenotype. After 7 days of differentiation, NGF was withdrawn from the medium to induce apoptosis and the cells were maintained for up to 4 days for protein extraction and determination of cell viability [36].

Induction of apoptosis in Jurkat T-cells

Human Jurkat T-cells (clone JE6-1) were obtained from ATCC (Rockville, MD, U.S.A.) and maintained in RPMI-1640 (Life Technologies, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, U.S.A.), 2 mM L-glutamine, 50 i.u./ml penicillin G and 50 μg/ml streptomycin. Jurkat cells were washed once in serum-free medium and suspended at 2×10^6 cells/ml for testing. Cell suspension (500 μl) was added to each well of 12-well culture plates, followed by the addition of 500 μl of 1 μM staurosporine (Calbiochem, La Jolla, CA, U.S.A.) or 600 ng/ml anti-Fas (Upstate Biotechnology, Lake Placid, NY, U.S.A.) in RPMI medium [7]. Cells were maintained at 37 °C in a 5% CO_2 atmosphere and harvested at 3, 6 and 24 h for protein extraction and determination of cell viability.

Assaying for ICE and CPP32 activities in apoptotic granule neuron extracts

Cultures were washed three times with serum-free medium and resuspended in 1 ml of TBS/EDTA buffer [20 mM Tris/HCl (pH 7.4 at room temperature)/155 mM NaCl/1 mM EDTA]. Cells from three wells were scraped and collected into 15 ml tubes and centrifuged for 5 min at 4 °C at 3600 ***g***. Cell pellets were resuspended and lysed in a buffer containing 20 mM Tris/HCl (pH 7.4 at 4 °C), 150 mM NaCl, 1 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA and 1% (w/v) Triton X-100 for 1 h. The lysates were cleared by centrifugation and stored at −70 °C in 50% (v/v) glycerol. To assay ICE and CPP32 proteolytic activities, Ac-YVAD-MCA and Ac-DEVD-MCA (where MCA represents 7-amido-4-methylcoumarin) were used (Bachem Bioscience, King of Prussia, PA, U.S.A.), respectively. Cell lysate was added to a buffer containing 100 μM peptide substrate, 100 mM Hepes, 10% (v/v) glycerol, 1 mM EDTA, 10 mM dithiothreitol and 10 μM Z-D-DCB or other protease inhibitor (optional). Fluorescence (excitation at 380 ± 15 nm and emission at 460 ± 15 nm) was measured every 15–30 min up to 60 min with a Millipore Cytoflor 2300 fluorescence plate-reader.

α-Spectrin digestion *in vitro* with ICE homologues and calpains

Mature forms of ICE, ICH-1, ICH-2 and CPP32 (β form) were cloned and functionally expressed as His-tagged proteins by *Escherichia coli* essentially as previously described [8,37]. Total protein was extracted from six 12-well plates of resting granule neurons by using the Triton X-100 method described above and used as a source of α-spectrin. Granule cell protein extract (20 μg of protein) was subjected to 2.5 μg of purified recombinant ICE, ICH-1, ICH-2 or CPP32 (β form), or 1.5 μg of purified μ-calpain or m-calpain [38] in 100 mM Hepes buffer (pH 7.2 at 25 °C)/10 mM dithiothreitol/10% (v/v) glycerol/1 mM EGTA for 90 min. The digestion was neutralized by the addition of an equal volume of SDS-containing sample buffer for PAGE. Samples were split into two sets. Both sets were subjected to electrophoresis and electrotransfer. The blots were then probed with anti-α-spectrin and anti-SBDP150 respectively (where SBDP stands for α-spectrin breakdown product).

Cell death measurement

SH-SY5Y cell death was assessed by measuring the cytosolic enzyme, lactate dehydrogenase, released into the medium (25 μl samples) as described earlier [39]. To measure cell death in granule neurons, 40 μg/ml propidium iodide was added directly to the wells at 24 h and fluorescence of the dye–DNA complex (excitation at 530 nm, emission at 620 nm) was measured after 5 min with a Millipore Cytoflor 2300 fluorescence plate-reader.

Hoechst staining of apoptotic nuclei

Cells were washed twice with PBS and then labelled with 1 μg/ml of the DNA dye Hoechst 33258 (bis-benzimide; Sigma) in PBS for 5 min at room temperature, using enough solution to cover

the cells completely. The cells were rinsed twice with PBS and then observed on a Leica DMIL fluorescence microscope with a UV2A filter.

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 analysed for protein concentration with a modified Lowry assay (Bio-Rad). Equal amounts of total protein (15 μg) were loaded on each lane and run on SDS/PAGE [4–20% (w/v) acrylamide] with a Tris/glycine running buffer system and then transferred to a PVDF membrane (0.2 μm) with a Tris/glycine buffer system with a semi-dry electrotransferring unit (Bio-Rad) at 20 mA for 1.5–2 h. The blots were probed with an anti-α-spectrin (monoclonal, Chemicon) antibody, anti-PEST (where PEST is a protein sequence that is enriched with proline, glutamate/aspartate, serine and threonine) and anti-SBDP150 (both polyclonal; gifts from Dr. Takaomi Saido, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Anti-m-calpain (polyclonal antibody; a gift from Dr. T. Shearer, Oregon Health Science University, Portland, OR, U.S.A.), anti-μ-calpain (polyclonal C25-1.1.1; a gift from Dr. R. Nixon and Dr. F. Grynspan) anti-[small subunit of calpain (s-calpain)] (clone P1, developed by Dr. R. Mellgren, purchased from Chemicon), anti-ICE (a gift from Dr. Brenda Shivers, Parke-Davis) [41], anti-CPP32 and anti-ICH-1 (both monclonal; Transduction Laboratories) and 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 with a colour scanner (Umax UC630) and the NIH program Image 1.5.

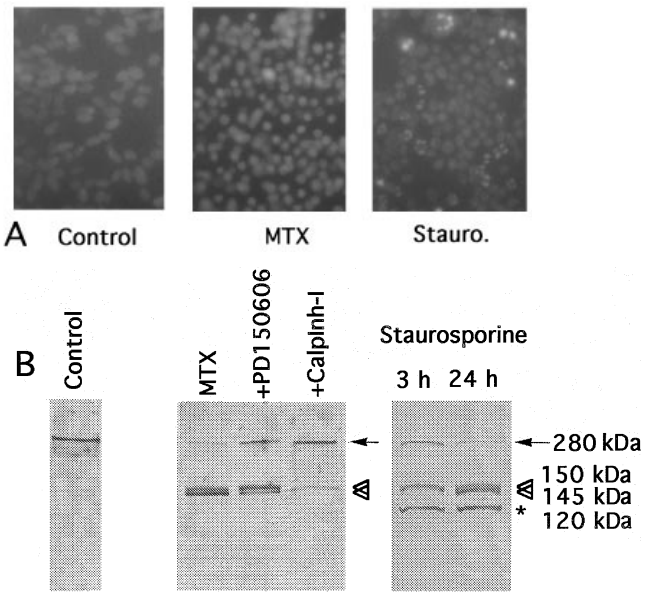

Figure 1 Different patterns of α-spectrin breakdown in necrotic and apoptotic SH-SY5Y cells

(**A**) SH-SY5Y cells were either untreated or challenged with 0.01 nM MTX for 3 h to induce necrosis or with 0.5 μM staurosporine for 5 h to induce apoptosis. Hoechst stain 33258 was used to stain nuclear DNA. (**B**) SH-SY5Y cultures were either untreated (Control) or subjected to either 0.5 μM staurosporine for 3 or 24 h, or to 0.1 nM MTX, in the absence or the presence of 10 μM PD150606 or CalpInh-I for 3 h (as indicated). Total cellular protein was extracted and analysed by PAGE (15 μg of protein per lane), electrotransferred and then probed with anti-α-spectrin antibody. Shown here is a developed blot. The solid arrow indicates the α-spectrin protein, the open triangles indicate the SBDP doublet of 150 and 145 kDa, and the asterisk indicates the apoptosis-specific 120 kDa SBDP. Results shown are representative of at least three experiments.

RESULTS

Different α-spectrin breakdown patterns in apoptotic and necrotic cells

To study proteolytic events specific to apoptosis rather than necrosis, we first established conditions to produce the two different forms of cell death. Human neuroblastoma SH-SY5Y cells, when treated with staurosporine, produced a classic apoptotic response, including chromatin condensation on the margin of nuclear envelope (shown with Hoechst 33258; Figure 1A), DNA laddering and formation of apoptotic bodies (results not shown). In contrast, when SH-SY5Y cells were challenged with MTX, we obtained a robust necrotic response: irregular scattering of condensed chromatin (Figure 1A), calcium overload, cell swelling and non-specific DNA fragmentation (results not shown).

In MTX-treated SH-SY5Y cells, α-spectrin (280 kDa) was cleaved by endogenous calpains to produce two characteristic fragments of 150 and 145 kDa (SBDP150 and SBDP145) as well as the loss of the intact α-spectrin, as has been reported previously [34,42,43] (Figure 1B). Both SBDPs were effectively blocked by CalpInh-I whereas PD150606 blocked the slower-forming SBDP145 (Figure 1B), as reported previously [34]. In contrast, challenging SH-SY5Y cells with staurosporine resulted in three SBDPs of 150, 145 and 120 kDa whereas intact α-spectrin diminished (Figure 1B). (The intact α-spectrin did not transfer efficiently owing to its size whereas the SBDPs did. Thus it gave rise to the impression that there were more SBDPs than intact spectrin to start with.) The formation of SBDPs in fact followed the time course of apoptosis closely. The presence of the SBDP150/SBDP145 doublet would indeed suggest that calpain is activated under apoptotic conditions. The presence of an intense SBDP120 band, however, appears to be unique to apoptosis.

Next, in cerebellar granule neurons undergoing spontaneous apoptosis in low-K^+ medium [44], we observed that the 150 and 145 kDa SBDP doublet and an SBDP120 band were formed whereas the intact α-spectrin level was decreased (Figure 2A). In contrast, MTX treatment of granule neurons produced only SBDP150 and SBDP145. In addition, a very similar SBDP pattern was found in two other distinct cell types undergoing apoptosis: rat PC-12 cells (differentiated) 3 days after NGF withdrawal [36] and human Jurkat T-cells after being challenged with either staurosporine or with an anti-Fas antibody [7] (Figures 2B and 2C). In the last case, almost all intact α-spectrin was degraded and SBDP145 seemed to be less noticeable.

Calpain and ICE-like protease inhibitors alter the α-spectrin breakdown pattern

In apoptotic granule cells, 20 μM CalpInh-I increased the level of intact α-spectrin and completely blocked the formation of SBDP145 whereas SBDP150 seemed to be enhanced (Figure 3A). However, CalpInh-I had no effect on SBDP120 (Figure 3A). We therefore examined the potential effect of ICH inhibitor Z-D-DCB and found that at 30 μM it effectively inhibited SBDP120 formation and increased the level of intact α-spectrin (Figure 3A). Moreover, a combination of Z-D-DCB and

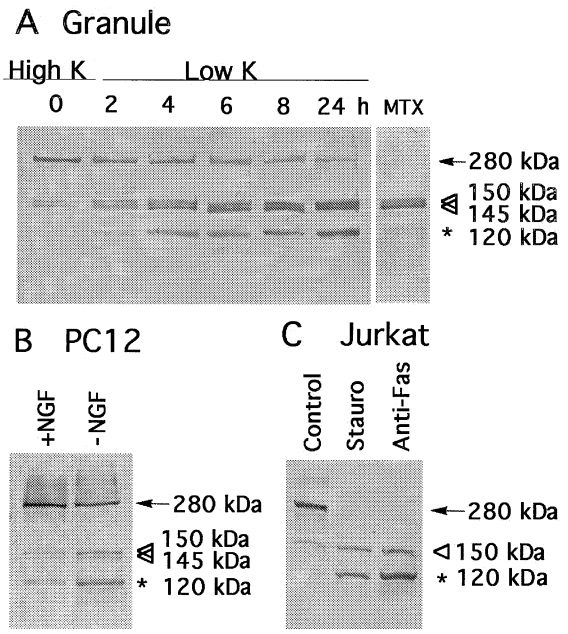

Figure 2 Fragmentation pattern for α-spectrin in apoptotic granule neurons (A), PC-12 (B) and Jurkat T-cells (C)

(**A**) Granule neurons were either maintained in high-K^+ medium for 24 h or exposed to low-K^+ medium for various lengths of time. (**B**) PC-12 cultures were maintained with (+NGF) or without (−NGF) NGF for 3 days. A basal level of SBDPs was observed as a reflection of background cell death. (**C**) Jurkat T-cells were either untreated or treated with 0.5 μM staurosporine (Stauro) or with 300 ng/ml anti-Fas antibody (Anti-Fas) for 4 h. Labelling for α-spectrin protein and SBDPs on the Western blots was as described in the legend to Figure 1. Results shown are representative of three experiments.

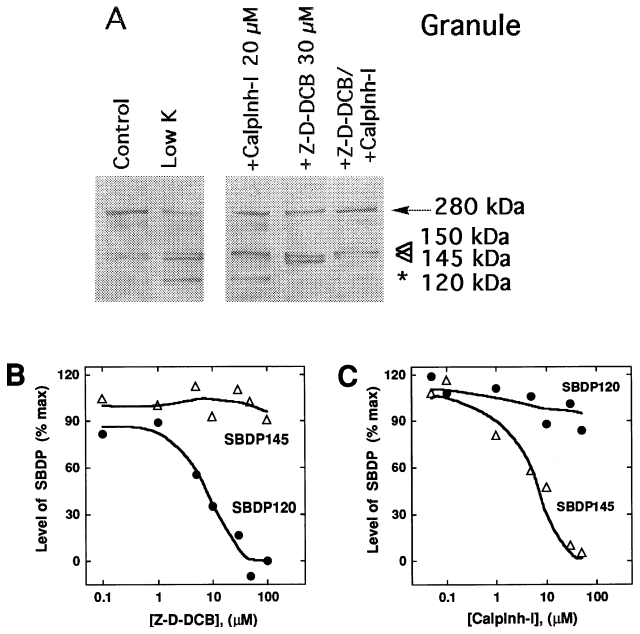

Figure 3 Effects of inhibitors of calpain and ICE-like proteases on SBDP pattern in apoptotic granule neurons

(**A**) Granule neurons were either maintained in medium with high K^+ (Control) or subjected to low K^+ alone or in the presence of either 30 μM Z-D-DCB or 20 μM CalpInh-I, or a combination of both inhibitors (as indicated). (**B**, **C**) SBDP120 (●) and SBDP145 (△) levels in apoptotic granule cells in the presence of various concentrations of Z-D-DCB (**B**) or CalpInh-I (**C**) were measured densitometrically and calculated as percentages of maximum (as in low K^+ alone). Results shown are representative of at least two experiments.

CalpInh-I blocked the formation of both SBDP145 and SBDP120 and enhanced the level of intact α-spectrin. Some residual SBDP150 was always observed despite the presence of both inhibitors, probably because the cleavage region of α-spectrin is highly sensitive to protease [45]. The specificity of CalpInh-I and Z-D-DCB towards the decrease in SDBP120 and SBDP145 were further quantified. CalpInh-I selectively decreased SBDP145 with an EC_{50} of 7.0 μM but had no effect on SBDP120 (Figure 3C). Similarly, SBDP145 was not observed if extracellular calcium was removed (results not shown). In contrast, Z-D-DCB decreased SBDP120 with an EC_{50} of 6.5 μM but had no effect on SBDP145 (Figure 3B). Similar results were obtained in staurosporine-treated SH-SY5Y cells (results not shown).

Protease inhibitors with diverse specificity were further tested in apoptotic granule cells against SBDP120 formation (Table 1). Lysosomal pH-neutralizing agents (NH_4Cl and chloroquine), the aspartic protease inhibitor pepstatin and the metalloprotease inhibitor 1,10-phenanthroline showed either a small decrease or no effect on the overall level of SBDP120 (Table 1). The serine protease inhibitor 4-(2-aminoethyl)-benzenesulphonylfluoride seemed to increase the level of intact spectrin partly and decreased SBDP120, consistent with previous reports that a serine protease might be involved in apoptosis [14,15]. Whereas CalpInh-I was ineffective, Z-D-DCB completely blocked SBDP120. In addition, another ICH inhibitor, Z-VAD(OEt)-DCB, that inhibits ICE and CPP32 (results not shown) also blocked SBDP120. Lastly, the CPP32-preferring Ac-DEVD-H provided a partial blockade whereas the ICE-preferring Ac-YVAD-H was essentially ineffective (Table 1).

Table 1 Effects of various protease inhibitors on SBDP120 level in apoptotic granule cells

Granule neurons were subjected to low K^+ alone (LK) for 16 h or in the presence of various protease inhibitors. SBDP120 was quantified by densitometry on Western blots. Results shown are averages from two experiments. Abbreviation: AEBSF, 4-(2-aminoethyl)-benzenesulphonylfluoride.

Treatment	SDBP120 level (% of LK)
Low K^+	100
+10 mM NH_4Cl	80
+50 μM chloroquine	71
+50 μM pepstatin	100
+50 μM 1,10-phenanthroline	95
+20 μM CalpInh-I	91
+1 mM AEBSF	53
+20 μM Z-D-DCB	4
+10 μM Z-VAD(OEt)-DCB	6
+100 μM Ac-DEVD-H	48
+100 μM Ac-YVAD-H	87

Activation of calpain and CPP32 in apoptotic cells

Anti-μ-calpain detected the intact protein in resting granule cells whereas in apoptotic cells the presence of both the intact 80 kDa form and the activated 76 kDa form was detected [46] (Figure 4A). Anti-m-calpain detected the presence of the intact 80 kDa

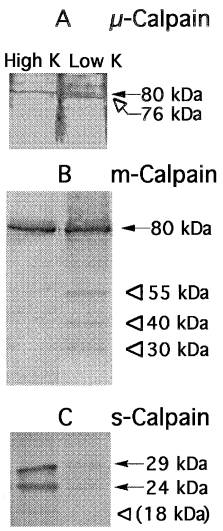

Figure 4 Evidence of autolysis of μ-calpain (A), m-calpain (B) and s-calpain (C) in apoptotic granule neurons

Granule neuron cultures were either untreated (control, left-hand lane) or challenged with low-K^+ medium for 16 h (right-hand lane). Total cellular proteins were extracted and analysed by PAGE (15 μg of protein per lane), electrotransferred and then probed with antibody against (**A**) μ-calpain, (**B**) m-calpain or (**C**) s-calpain. The solid arrow indicates the intact protein and autolysis products are indicated by open triangles. In (**C**), the 18 kDa fragment was non-antigenic and was thus not observed. Results shown are representative of at least three experiments.

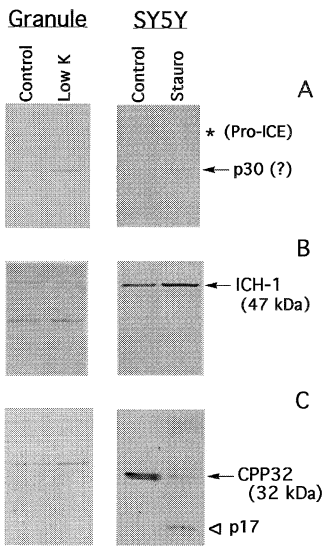

Figure 5 Detection of ICE-like proteases in SH-SY5Y cells and granule neurons

SH-SY5Y (SY5Y) cultures were either untreated (Control) or subjected to 0.5 μM staurosporine alone (Stauro). As indicated, granule neurons were either maintained in high-K^+ medium (Control) or subjected to low K^+ for 16 h. Total cellular proteins were extracted and analysed by PAGE (15 μg of protein per lane), electrotransferred and then probed with antibody against (**A**) ICE, (**B**) ICH-1 or (**C**) CPP32 (as indicated). Solid arrows indicate the intact proteins, and in (**C**) the autolysis product (p17) of CPP32 is indicated by an open triangle. In (**A**), an asterisk indicates the predicted position of pro-ICE (45 kDa) which was not observed. The p30 band is a non-specific protein detected by the antiserum. Results shown are representative of at least three experiments.

m-calpain in resting cells and there was partial conversion to autolytic fragments of 55, 40 and 30 kDa in apoptotic cells [47] (Figure 4B). Also the anti-s-calpain detected the intact subunit (29 kDa band) and a partly degraded form (24 kDa) in untreated granule cells. These forms of s-calpain disappeared in apoptotic cells, suggesting that the s-calpain was autolytically transformed to the activated 18 kDa form, which was not detected by this antibody, as described before [48] (Figure 4C). Together, these results show that both calpain isoforms are present in granule neurons and are activated during apoptosis.

An anti-ICE serum did not detect any pro-ICE (45 kDa) signal in either cell type, except a weak non-specific protein at approx. 30 kDa (Figure 5A). [The 30 kDa protein was also detected in ICE knockout mice (B. Shivers, unpublished work) and thus it did not originate from ICE.] Anti-(human ICH-I) antibody detected the intact protein (47 kDa) in the control SH-SY5Y cells and somewhat more intensely in apoptotic SH-SY5Y cells. However, no sign of autolytic fragmentation was observed. [The anti-ICH-1 antibody was raised against the C-terminal third (residues 225–401) of human ICH-1 and can detect the C-terminal fragment generated by autolysis.] The antibody barely detected the rat protein in granule neurons, perhaps as a result of amino acid differences between species (Figure 5B). Lastly, an anti-CPP32 (human) antibody detected the intact protein (32 kDa) in control SH-SY5Y cells. In apoptotic SH-SY5Y cells the intact protein was much decreased and was replaced by a 17 kDa autolytic fragment (p17 in Figure 5C). Apparently the anti-human CPP32 antibody did not cross-react with rat CPP32.

As an alternative approach to ICE homologue activation in granule cells, Triton X-100 lysate at different time points of potassium withdrawal was assayed with Ac-DEVD-MCA and Ac-YVAD-MCA. CPP32 strongly prefers Ac-DEVD-MCA over Ac-YVAD-MCA, whereas ICE cleaves both at a similar rate (R. V. Talanian, unpublished work) [13]. We observed that whereas the Ac-YVAD-MCA-hydrolytic activity remained very low throughout the time course, Ac-DEVD-MCA-hydrolytic activity was increasing progressively on K^+ withdrawal and elevated to more than 30-fold the basal activity at 16 h (Table 2). The hydrolytic activities against Ac-YVAD-MCA and Ac-DEVD-MCA remained very low in cells maintained in high K^+ for 16 h. When we reassayed the cell lysate (LK, 16 h) in the presence of Z-D-DCB, CalpInh-I and PD150606, only Z-D-DCB effectively inhibited this activity (Table 2).

Characterization of the SBDPs produced by calpain and ICE homologues *in vitro* and *in situ*

Protein extracts from control granule neurons were also treated directly with recombinant and purified ICE, ICH-1, ICH-2, CPP32 (β form) and purified μ-calpain and m-calpain. On an immunoblot probed with an anti-α-spectrin antibody, we observed that each of the four members of the ICE family tested could reduce the level of intact α-spectrin and produced an SBDP of 150 kDa (Figure 6A). In addition, CPP32 and ICE (to a lesser extent) could produce an SBDP of 120 kDa. In contrast, μ-calpain produced SBDP145 whereas m-calpain produced both SBDP150 and SBDP145. μ-Calpain could also produce SBDP150 with a shorter incubation time (result not shown). When CPP32 and calpain were both used, we found both SBDP145 and SBDP120 (Figure 6A). We also used an antibody (anti-SBDP150) that recognizes only the new N-terminus (GMMPR) of the 150 kDa SBDP produced by calpain [42]. The 150 kDa SBDP produced by ICHs was not detected by anti-SBDP150 whereas

Table 2 Measurement of ICE-like protease activities in apoptotic granule neurons

Granule neurons were subjected to medium with either high K^+ (HK) for 0 or 16 h or low K^+ (LK) for 0–16 h. Cell lysates were assayed with Ac-YVAD-MCA or Ac-DEVD-MCA. For the 16 h time point (LK only), the effects of 10 μM Z-D-DCB (D), PD150606 (PD) or CalpInh-I (CI) were also tested. Values are means ± S.E.M. ($n = 3$).

	Ac-DEVD-MCA hydrolysis (fluorescence unit)							
Time (h) ...	0	3	5	8	16	16 + D	16 + PD	16 + CI
LK	6.7 ± 1.7	41.3 ± 2.9	144 ± 26	177 ± 12	263 ± 8	1.0 ± 0.4	226 ± 37	257 ± 39
HK	–	–	–	–	11.7 ± 0.9	–	–	–
	Ac-YVAD-MCA hydrolysis (fluorescence unit)							
Time (h) ...	0	3	5	8	16			
LK	14.3 ± 0.9	17.7 ± 1.7	4.7 ± 0.8	19.0 ± 2.6	8.7 ± 0.5			
HK	–	–	–	–	5.0 ± 1.0			

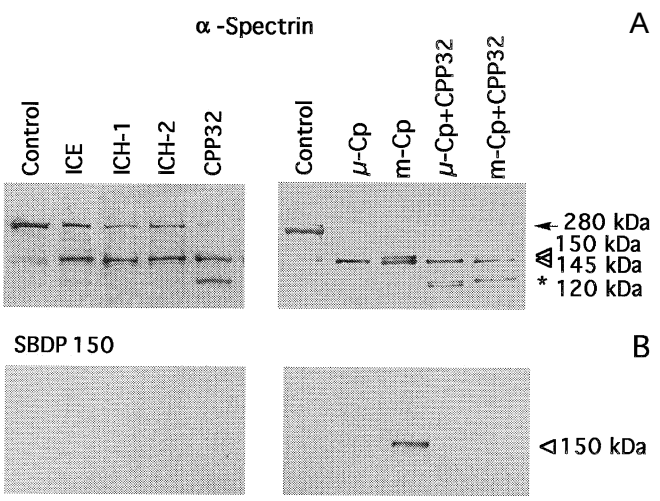

Figure 6 Fragmentation of α-spectrin by ICHs and calpains *in vitro*

Granule cell protein extract (20 μg of protein) was treated with ICE, ICH-1, ICH-2 or CPP32, or μ-calpain (μ-Cp) or m-calpain (m-Cp). The samples were analysed on Western blots probed with anti-α-spectrin (**A**) or anti-SBDP150 (**B**). Labelling for α-spectrin protein and SBDPs is described in the legend to Figure 1. Results shown are representative of three experiments.

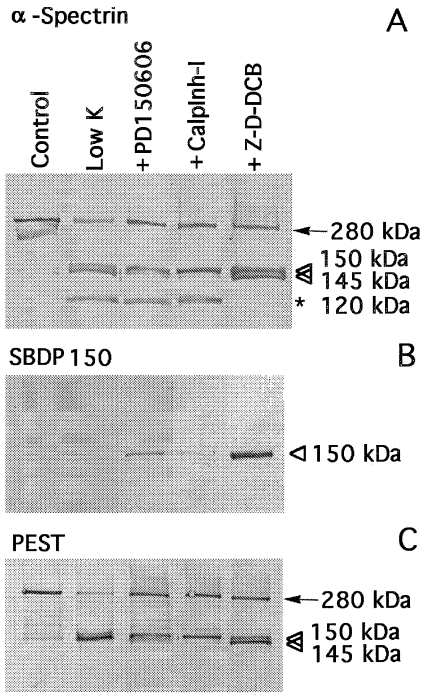

Figure 7 Characterization of SBDPs in apoptotic granule neurons with three α-spectrin antibodies

Granule neurons were either maintained in high-K^+ medium (Control) or subjected to low K^+ alone for 16 h or in the presence of PD150606 (5 or 20 μM), CalpInh-I (5 or 20 μM) or Z-D-DCB (10 or 50 μM). Samples were split into three sets and each set was subjected to PAGE and electrotransfer. The immunoblots were probed with anti-α-spectrin (**A**), anti-SBDP150 (**B**) or anti-PEST antibody (**C**). Labelling for α-spectrin protein and SBDPs are as described in the legend to Figure 1. Results shown are representative of two experiments.

the SBDP150 produced by m-calpain was detected (Figure 6B). These results suggest that, although nearly identical in electrophoretic mobility, the ICH-produced 150 kDa fragment (now called SBDP150i) and the calpain-produced SBDP150 have different N-termini. SBDP145 or SBDP120 were not detected by anti-SBDP150.

We further characterized the various SBDPs produced in apoptotic granule neurons *in situ*. In the low-K^+-treated cells, intact α-spectrin was decreased and gave way to SBDPs of 150, 145 and 120 kDa (Figure 7A, lane labelled Low K). We found that anti-SBDP150 did not detect any of three fragments (Figure 7B, lane labelled Low K). An anti-SBDP150-detectable fragment was observed only when either an ICH inhibitor or a calpain inhibitor was used (Figure 7B). At 20 μM, CalpInh-I inhibited significantly the formation of SBDP150 (Figure 7B). Thus the 150 kDa band still observed in Figure 7A was probably produced by ICH. Furthermore we used another antibody (anti-PEST) that recognizes the single PEST sequence in α-spectrin, which is located C-terminal to the initial calpain cleavage [42]. Anti-PEST detected the intact α-spectrin, SBDP150/SBDP150i and SBDP145 but not SBDP120 (Figure 7C).

Anti-apoptotic effects of ICH and calpain inhibitors

It is tempting to suggest that α-spectrin degradation and the resultant disruption of cytoskeletal network might be necessary

Table 3 Anti-apoptotic effects of ICH and calpain inhibitors in SH-SY5Y cells and granule neurons

In (**a**), SH-SY5Y cells were either untreated (Control) or subjected to 0.5 μM staurosporine alone (Staurosporine) or in the presence of 100 μM Z-D-DCB, 20 μM CalpInh-I or 25 μM PD150606. Values are means ± S.D. ($n = 4$). In (**b**), granule neurons were either maintained in high-K^+ medium or subjected to low K^+ alone for 16 h or in the presence of Z-D-DCB (30 μM), CalpInh-I (20 μM) or both Z-D-DCB and CalpInh-I. Values are means ± S.D. ($n = 6$). Data significantly different (tested by Student's t test) from staurosporine alone (**a**) or low K^+ alone (b) are indicated by * or †($P < 0.0005$) or by ** or †† ($P < 0.0001$) respectively.

(a) SH-SY5Y cells	Cell death (LDH release; units/ml)
Control	163.4 ± 18.9
Staurosporine	573.6 ± 72.3
+ 100 μM Z-D-DCB	157.9 ± 16.3**
+ 20 μM CalpInh-I	368.1 ± 56.4*
+ 25 μM PD150606	348.3 ± 78.9*
(b) Granule neurons	**Cell death (fluorescence units)**
High K^+	95.3 ± 20.5
Low K^+	281.7 ± 37.0
+ 30 μM Z-D-DCB	175.6 ± 33.0†
+ 20 μM CalpInh-I	148.3 ± 11.9†
+ Z-D-DCB + CalpInh-I	93.7 ± 24.7††

steps in the expression of apoptosis. In the SH-SY5Y model, Z-D-DCB at 100 μM indeed provided almost an complete inhibition of apoptotic death (Table 3). A lower concentration (50 μM) of Z-D-DCB also provided significant but less protection (results not shown). SH-SY5Y cells could apparently tolerate these concentrations of Z-D-DCB alone quite well (results not shown). The protective effects of Z-D-DCB were confirmed by both a decrease in the number of apoptotic nuclei and an inhibition of DNA fragmentation (R. Nath, K. J. Raser, D. Stafford and K. K. W. Wang, unpublished work). CalpInh-I (20 μM) as well as PD150606 (25 μM) also provided partial anti-apoptotic effects (Table 3). Their ability to decrease the number of apoptotic nuclei, however, was only marginal (results not shown). In the granule-cell apoptosis model, both Z-D-DCB (30 μM) and CalpInh-I (20 μM) provided almost the same level of apoptosis reduction (Table 3). Under these conditions, both Z-D-DCB and CalpInh-I decreased the number of apoptotic nuclei, as determined by Hoechst 33258 staining (R. Nath, K. J. Raser, D. Stafford and K. K. W. Wang, unpublished work). Combined treatment with both inhibitors provided the best protection against apoptosis.

DISCUSSION

In this study we have provided evidence that α-spectrin is degraded in neuronal cells that undergo apoptosis. However, unlike in necrosis, we found for the first time that α-spectrin is not only cleaved by calpains but also by one or more members of the ICE-like protease family (ICHs) during apoptosis. It has been reported that fragmented α-spectrin lacks the ability to interact with actin [45]. Thus spectrin cleavage by calpain and ICH can potentially lead to cytoskeletal derangement. It is also of interest to point out that α-spectrin binds to ankyrin, which contains a 'cell death' domain (similar to those found in tumour necrosis factor receptor and Fas/Apo1) [54]. Thus both calpain and ICH might participate in apoptosis by exerting their effects on their target proteins. Consistent with that, this is also the first study to demonstrate that calpain and ICE-like protease inhibitors can block neuronal apoptosis (Table 3).

We propose that calpain and ICH can each cleave α-spectrin at two sites. The previously identified initial calpain cleavage site VY↓GMMP is located within a sequence in repeat 11 and just N-terminal to the calmodulin-binding domain (VQQQEVYGMM-PRDETDSKTASPWKSARLMVHTVAFNSIK) [44,51–53]. We propose a putative sequence of DETD↓SK for the initial ICH cleavage because it is similar to the DEVD sequence found in PARP that is recognized by several ICHs [6,13]. The second calpain cleavage, which produces SBDP145, is as yet unidentified except that it has a different N-terminus from SBDP150 (Figures 6 and 7). The second ICH cleavage, which produced the SBDP120, must be C-terminal to the PEST sequence located between repeats 12 and 13 because it was not detected by anti-PEST (Figure 7). Consistent with that, the anti-SBDP150 also did not detect SBDP120 (Figures 6 and 7). This is in contrast with the study of Martin et al. [27] where they reported that a 120 kDa SBDP in apoptotic T-cells was detected by the anti-SBDP150 antibody. One technical difference was that, whereas we used a chromogenic method with alkaline phosphatase, they used an ^{125}I-autoradiographic method for the detection of the immunoreactive bands. It is possible that the autoradiographic method was so sensitive that it might have detected a low level of cross-reactivity between the anti-SBDP150 and the SBDP120, although the latter does not necessarily contain the free N-terminal GMMPR found in SBDP150. We have also found that purified α-spectrin is also fragmented by CPP32 at two sites, producing immunoreactive fragments of 150 and 120 kDa (results not shown). Work is currently in progress in our laboratories to identify the exact ICH cleavage sites by N-terminal sequencing. Because the SBDP120 is apoptosis-specific regardless of cell type, but is absent in cell necrosis (Figures 1 and 2), we propose that SBDP120 can be used as a protein marker for apoptosis.

Regarding the ICE homologue(s) involved, we did not detect ICE in either cell type. Both cell types also lack the ICE mRNA message ([49]; K. M. Keane and B. D. Shivers, unpublished work). However, our results clearly suggest that CPP32 or a CPP32-like protease (e.g. Mch3) is present and is activated during apoptosis in both cell types (Figure 5 and Table 2). Furthermore SBDP120 was most efficiently produced by CPP32 in α-spectrin digestion *in vitro* (Figure 6). As all ICE homologues recognize an Asp residue in the P1 position in their substrates, a previously identified aspartate-based ICE inhibitor Z-D-DCB [13] could indeed inhibit CPP32 (results not shown), is cell permeable [50] and lacks significant effects on calpains (up to 100 μM; results not shown). We found that Z-D-DCB was very effective in blocking SBDP120 formation. The other two effective ICH inhibitors, Z-VAD(OEt)-DCB and Ac-DEVD-H, are also good inhibitors for CPP32 (Table 1). The more ICE-specific inhibitor YVAD-H did not block SBDP120.

In parallel we also confirmed the presence of autolytic activation of both calpain isoforms in neuronal apoptosis (Figure 4). CalpInh-I also attenuated apoptosis in granule neurons (Table 3). This is consistent with recent reports that calpain inhibition can block apoptosis in T-cells and thymocytes [19–21]. In apoptotic SH-SY5Y cells, autolytic activation for calpains was less pronounced (results not shown). We suspect that calcium increases might be more confined to certain local subcellular areas in this cell line. The anti-apoptotic effects of calpain inhibitors in SH-SY5Y cells were only partial whereas ICH inhibition could provide almost complete protection (Table 3). Furthermore, under conditions where there was little calpain activation (i.e. in the absence of extracellular Ca^{2+}), apoptosis could apparently still occur (R. Nath and K. K. W. Wang,

unpublished work). Thus it is possible that SH-SY5Y cells can undertake an apoptotic pathway in which calpain activation is not absolutely required. One hypothesis that can explain the different results in different models is that calpain and ICH might attack the same or similar substrates (e.g. α-spectrin) during apoptosis. This redundant mechanism would allow either protease alone or both in combination to activate the apoptotic machinery. Thus different cell types could conceivably adopt somewhat different pathways of apoptosis. Further work in this area should allow us to test this hypothesis.

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