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Calpain inhibitors, but not caspase inhibitors, prevent actin proteolysis and DNA fragmentation during apoptosis

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SUMMARY

Apoptosis, or programmed cell death, involves a cascade of regulatory events leading to the activation of specific proteases. However, the key substrates for these proteases remain to be identified. We previously demonstrated that levels of five unidentified polypeptides were specifically increased in neurons from embryonic chicken ciliary ganglia undergoing apoptosis by trophic deprivation. Here we show by microsequencing of two of these polypeptides that they are fragments of actin. One of them represents cleavage of actin at the site of interaction with DNase I. The same actin fragments are also found at early stages of apoptosis in chicken and rat dorsal root ganglion neurons, chicken spinal motoneurons and rat thymocytes. Actin fragmentation may play a role in the apoptotic process, since calpain inhibitors I and II both inhibit neuronal death and suppress actin fragmentation. In contrast, caspase (ICE family) inhibitors, though effective in delaying neuronal death, do not prevent actin cleavage or DNA fragmentation. These results indicate a key role for calpain-like proteases in neuronal programmed cell death and suggest that actin fragmentation in the cell is correlated with subsequent DNA fragmentation.

Key words: Neuronal cell death, Apoptosis, Calpain

INTRODUCTION

Programmed cell death (PCD) occurs in many cell types and organs during ontogenesis. This cell death is especially important in the nervous system (for review, see Henderson, 1996) and has been particularly well studied in the peripheral nervous system (Levi-Montalcini, 1964) and in the spinal cord (Oppenheim, 1985), where up to 50% of the neurons die during a restricted period, at developmental stages that vary according to the tissue and species. For example, the chicken ciliary ganglion loses 50% of the neurons between day 8 and 14 of the embryonic life (Oppenheim, 1985). Several experimental data suggest that this cell death serves, after a period of over-production, to adjust the final number of neurons to that necessary for optimal innervation of their target tissue (Cowan et al., 1984; Oppenheim, 1991).

One important breakthrough in understanding the molecular mechanisms of PCD was the discovery in vertebrates of death-inducing and death-blocking genes analogous to the death genes characterized in the nematode *C. elegans* by the group of Horvitz (for a review, see Ellis et al., 1991; Ellis and Horvitz, 1986; Hengartner et al., 1992). The product of *ced-3*, a death-inducing gene of *C. elegans*, was found to be homologous to the interleukin-1β-converting enzyme (ICE), a cysteine protease (Miura et al., 1993). Several other members of the ICE family, now named 'caspases' (Alnemri et al., 1996) because they share a cysteine residue at their active site and cleave after aspartate residues, have since been identified (for review, see Takahashi and Earnshaw, 1996; Martins and Earnshaw, 1997). The death-inducing potential of ICE (caspase-1) was shown first in vitro, either by introducing expression vectors carrying the *ice* gene into cells, thereby killing them, or by blocking cell death with an inhibitor of ICE, the viral serpine CrmA (Miura et al., 1993). Milligan et al. (1995) demonstrated that caspase-1 (or members of the caspase family) may play a role during normal developmental cell death. They showed that PCD of lumbar spinal motoneurons or interdigital cells could be blocked by inhibitors of the caspase family. ICE itself is probably not the active enzyme, because knock-out mice for *ice* do not present any perturbation in the PCD occurring in the nervous system (Kuida et al., 1995; Li et al., 1995). However, null mutants for CPP-32 (caspase-3) present a number of defects in the development of the nervous system (Kuida et al., 1996). In particular, too many neurons are present in the brain, and the mice die at early postnatal stages. Caspase-3 therefore seems likely to play an important function in the regulation of cell death in the mammalian nervous system.

The involvement of the caspase family in the cell death process does not exclude a role for other families of protease, such as the calpains or proteasome complexes. Activation of

calpains is an early event in the onset of thymocyte cell death (Squier et al., 1994) as well as in the death of CA-1 pyramidal neurons after NMDA receptor hyperstimulation (Roberts-Lewis et al., 1994). The proteasome has been shown to be involved in neuronal death induced by trophic deprivation (Sadoul et al., 1996).

Although much progress has been made in the identification of the different proteases involved in cell death, the characterization of their major substrates, whose proteolysis is supposed to lead to cell death, remains incomplete. As well as hydrolysing pro-interleukin-1β in the case of caspase-1, the caspases can auto- and/or cross-proteolyse each other. Most other substrates identified thus far belong to two main categories. In the first are DNA repair enzymes such as poly(ADP-ribose) polymerase (PARP) (Lazebnik et al., 1994) and the catalytic subunit of the DNA-dependent protein kinase (Casciola-Rosen et al., 1995; Song et al., 1996). In the second category are structural proteins of the nucleus or cytoskeleton, such as nuclear lamins and Gas-2 (Brancolini et al., 1995; Lazebnik et al., 1995). Not all of these proteins are necessarily physiological substrates of the proteases involved in apoptosis, as some were characterized using purified proteases (at high concentrations; Gu et al., 1995), on proteins, cell-free systems or processed cell lysates. For instance, actin proteolysis was initially shown to occur in apoptotic cells only after incubation of the cell lysate in a caspase-1 assay buffer (Mashima et al., 1995). More recently, however, actin cleavage was shown to occur in cultured neurotrophils undergoing spontaneous PCD (Brown et al., 1997).

As part of an attempt to identify physiological substrates of death-inducing proteases, we previously showed by 2-D PAGE analysis that the levels of five unidentified polypeptides were greatly increased in neurons from chicken ciliary ganglia cultured in conditions that would subsequently lead to apoptosis (Villa et al., 1994). Here, we show that two of the polypeptides that accumulate are proteolytic fragments of actin and that actin fragmentation is a general feature of pre-apoptotic cells. In searching to identify the enzyme responsible for actin proteolysis, we found that although both calpain inhibitors and general caspase inhibitors were able to support the survival of a fraction of the neurons, only the calpain inhibitors strongly reduced actin cleavage and DNA fragmentation. We propose a role for actin cleavage in the liberation of the DNase I that will lead to inter-nucleosomal DNA cleavage.

MATERIALS AND METHODS

Materials

Recombinant human ciliary neurotrophic factor (rh-CNTF) was a gift from SCIOS-NOVA (Mountain View, CA, USA). Insulin (I 5500), transferrin (T 1283), BSA (A 6003), progesterone (P 0130), selenium (S 5261), putrescine (P 5780) and poly-L-ornithine (P 3655) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM, 074-02100), fetal calf serum and trypsin-EDTA solution were from Gibco Laboratories (Grand Island, NY, USA). Tissue culture plastic dishes were purchased from Falcon (Becton Dickinson France SA). Acrylamide and LDS were purchased from Serva Feinbiochemica (Heidelberg, Germany), bis-acrylamide was purchased from Bio-Rad. The βmax films for autoradiography and [^{35}S]methionine (specific activity: 1,000 Ci/mmol) were from Amersham Corp. Calpain inhibitors were from Calbiochem (La Jolla, CA, USA) and the caspase inhibitors benzyloxycarbonyl-Val-Ala-Asp(Omethyl)-fluoromethylketone (Z-VAD-FMK) and t-butyloxycarbonyl-Asp(OMe)-fluoromethylketone (BAF) from Enzyme Systems Products (Dublin, CA, USA). TdT-mediated dUTP nick end labelling (TUNEL) staining was performed using the In situ cell death detection kit from Boehringer-Mannheim (Germany).

Cell cultures and evaluation of survival

Ciliary ganglion (cCG) and dorsal root ganglion neurons (cDRG) were cultured from 8-day-old (E8) Leghorn chicken embryos as described (Villa et al., 1994). Motoneurons (cMN) were purified from E5 chicken embryos and cultured as described (Bloch-Gallego et al., 1991; Camu et al., 1993). Rat dorsal root ganglion (rDRG) neurons were cultured from newborn Wistar rats using the same procedure as for chicken DRG.

Ganglion neurons were seeded at a density of 10,000 cells/cm^2 and motoneurons at 1,000 cells/cm^2, on polyornithine-laminin pre-treated tissue-culture plastic. All these neurons were seeded in serum-free medium (Henderson et al., 1995) and in the presence or absence of their respective survival factors: CNTF (20 ng/ml) for CG8, nerve growth factor (NGF, 50 ng/ml) for cDRG and rDRG and chicken muscle extract (10 μg protein/ml) for cMN. The chicken muscle extract was prepared as described by Bloch-Gallego et al. (1991). Treatments of cCG neurons with protease inhibitors were also performed at the time of seeding.

Rat thymocytes (rThy) were cultured from three-week-old Wistar rat thymuses, following the method described by Humblet et al. (1995). Thymocytes were cultured for 12 hours on tissue culture plastic at a density of 100,000 cells/cm^2, then 6 hours in the absence or in the presence of 10^{-6} M dexamethasone.

In all neuronal systems, cell survival was determined by direct visual counting after staining of the living cells with the vital dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Manthorpe et al., 1986). For rat thymocytes, survival was estimated after staining the dead cells with Trypan Blue.

Metabolic labeling

All neuronal cell types were cultured for 18 hours in the presence of 50 μCi/ml of [^{35}S]methionine (1,000 Ci/mmol). The radioactive medium was then removed and the cultures were washed three times with cold isotonic NaCl. Cells were scraped off with a rubber policeman, pelleted by centrifugation at 1,000 ***g*** for 10 minutes and stored at −80°C until analysis.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

Samples were prepared as described by Garrels (1979), modified by Loret et al. (1988). Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was performed as described by O'Farrell (1975), modified by Loret et al. (1988). Some modifications were added for the sequencing gel and are described below.

Silver staining and autoradiography of 2-D gels

Gels were fixed for 3 hours in 50% methanol and 12% trichloracetic acid, and then washed with several changes of distilled water. They were stained according to the method of Merril et al. (1981), and/or dried and exposed to Hyperfilm-βmax for times ranging from 3 to 15 days, depending on the radioactivity of the starting sample.

Preparation of gels and blots for microsequencing

For microsequencing, cell pellets of CNTF-untreated (pre-apoptotic) neuronal cultures (18 hours in vitro), derived from 8000 E8 ciliary ganglia, were pooled and the proteins extracted. This extract (about 1 mg of protein) was loaded on a 3 mm diameter tube gel, and the first dimension was performed. To help in subsequent identification of the

correct spots, a small percentage (about 1%) of metabolically labeled proteins extracted from apoptotic CG8 was added. The 1st dimension gel was then loaded on a 2nd dimension gel (2 mm thick). To minimize blocking of the N-terminal amino-acid, the urea for the 1st dimension gel was deionized and thioglycolic acid was added to the running buffer in the 2nd dimension. After 2-D PAGE, gels were blotted onto a PVDF membrane in the presence of DTT. The membrane was stained briefly with Coomassie blue to locate the spots. An autoradiogram was then obtained to confirm the localisation of the spots. Sequencing was performed using Edman protein sequence analysis (Henzel et al., 1994). One spot was sequenced directly from the PVDF membrane, the other one was first proteolysed with Lys-C, the fragments separated by HPLC and sequenced.

RESULTS

Two of the polypeptides that accumulate in pre-apoptotic ciliary ganglion neurons are fragments of actin

We cultured ciliary ganglion neurons from E8 chicken embryos (cCG) in the presence (Fig. 1a) or absence (Fig. 1b) of CNTF (20 ng/ml). After 18 hours in culture, most of the neurons remained alive and morphologically healthy in both conditions. As shown previously (Villa et al., 1994), the neurons cultured in the absence of CNTF start to die at about 20 hours and 50% of them are dead at 24 hours. When these cultures were metabolically labeled with [^{35}S]methionine and cell extracts analysed by 2-D PAGE, levels of 5 polypeptides, numbered arbitrarily from 1 to 5, with apparent molecular masses in the range 31 to 41 kDa were increased in pre-apoptotic neurons (Fig. 1d) when compared to neurons maintained by CNTF (Fig. 1c), as we previously reported (Villa et al., 1994). Two other non-variant spots in the same region of the gel, labeled A and B, were used as internal controls (Fig. 1c,d).

In order to determine the nature of the polypeptides that accumulated at the start of apoptosis, we performed large-scale cultures from a total of 8000 E8 ciliary ganglia. After transfer to PVDF membranes, each of the spots were submitted to microsequencing. Only two of the major spots yielded sequence data: spot 1 (41 kDa) and spot 2 (40 kDa). Quantities of spots 3, 4 and 5 were below the detection limits for sequencing, even at maximal loading of gels.

It was possible to directly microsequence spot 2 from its NH_2 terminus. This polypeptide corresponded to actin that had been cleaved after the Arg residue which is in position 37 in the case of β actin and in position 39 in the case of α actin (Fig. 2). Microsequencing of spot 1 from its NH_2 terminus was not possible. We therefore first performed a digestion of the polypeptide with Lys-C, directly on the blot, then isolated the fragments by HPLC and sequenced two of them. The sequences determined showed that this spot was also actin (Fig. 2), but it was of course not possible in this case to determine where proteolytic cleavage had taken place. The sequences obtained were found to be common to α, β or γ actin. This was expected for spot 2 since most of the differences between the three isoforms are located in the N-terminal decapeptide, which is absent from this fragment. For spot 1, the only difference in the corresponding published sequences of non-muscle actin (β and γ) and α actin is located at amino acid 129 (Vandekerckhove and Weber, 1979). This amino acid is a Thr in β and γ actin and a Val in α actin. During sequencing of spot 1, both Val and Thr were found in the peak corresponding to amino acid 129, suggesting that this spot contains both non-muscle and α actin, which has been shown to be present in the nucleus of neuronal cells (Milankov and De, 1993). However, as this spot was sequenced after enzymatic lysis with Lys-C, we cannot exclude that one of the two amino acids is generated by the autolysis of Lys-C.

Actin is also fragmented in pre-apoptotic chicken DRG neurons and motoneurons

To determine whether the accumulation of actin fragments was specific to cCG neurons, we looked for the appearance of Spots 1 and 2 in other neurons deprived of different neurotrophic factors. We compared two types of chicken neurons that have been shown to die by apoptosis: dorsal root ganglion (cDRG) neurons cultured in the absence of NGF (Allsopp et al., 1993) and spinal motoneurons cultured in the absence of muscle extract (Milligan et al., 1995).

After culture of the neurons for 18 hours in the absence or in the presence of their respective source of trophic support, in a medium containing 50 μCi/ml of [^{35}S]methionine, cell proteins were extracted and separated by 2-D PAGE, and the gel was subjected to autoradiography. The same actin fragments (Spots 1 and 2) were present at higher levels in cDRG neurons deprived of NGF (Fig. 1h), as compared to cDRG with NGF (Fig. 1g). A similar increase was observed in motoneurons deprived of muscle extract (Fig. 1l), relative to motoneurons with muscle extract (Fig. 1k). Actin fragmentation is thus a general feature in apoptotic chicken neurons.

Actin fragments accumulate during apoptosis of cells from other species and organs

Similar experiments were performed with purified neurons from DRG from newborn rat, which are highly dependent on NGF for their survival. Three polypeptide spots were more abundant in rat neurons cultured in the absence of NGF than in its presence. To confirm that these three polypeptides were the same in chicken and rat, we mixed a protein extract of apoptotic rDRG neurons with a trace amount of metabolically-labeled proteins from apoptotic cCG neurons. The mixture was submitted to 2-D PAGE and then silver-stained before autoradiography. By superimposing the silver-stained gel (Fig. 3a) and its autoradiogram (Fig. 3b), we showed that two of the spots in the rat extract present exactly the same physicochemical characteristics as spots 1 and 2 identified as actin fragments in chick. Therefore, the two polypeptides that accumulate in rat apoptotic cells are likely also to be fragments of actin.

To determine whether the actin fragments were specifically related to death induced by trophic factor deprivation, or whether they could also be generated in other apoptotic cells, we cultured dissociated rat thymocytes for 12 hours and treated them for 6 hours with 10^{-6} M dexamethasone (a potent inducer of apoptosis). Using Trypan blue as a test for viability, we determined that 75% of them had died after 18 hours in culture and 6 hours of treatment, while only 15% were dead in control cultures (data not shown). Rat thymocytes could not be submitted to metabolic radio-labelling, as these cells are very sensitive to radio-toxicity. Therefore, as in the previous paragraph, we mixed a protein extract of rat thymocytes

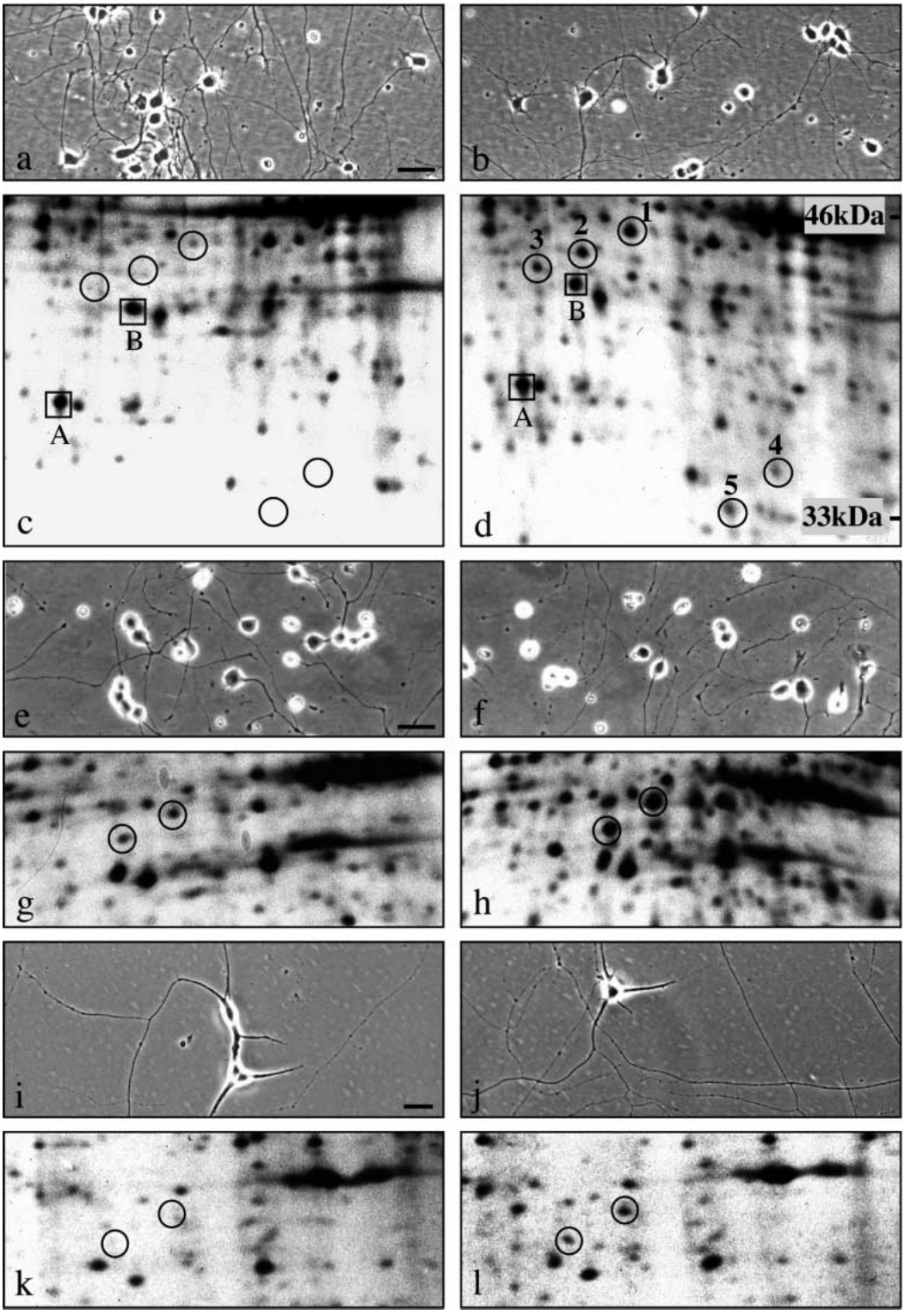


Fig. 1. The same two polypeptides accumulate in three different populations of apoptotic neurons. Embryonic chicken neurons were cultured for 18 hours in the presence or absence of their survival factors and in the presence of [^{35}S]methionine. Phase-contrast micrographs of cultured neurons (bar, 30 μm) are shown immediately above representative regions of autoradiograms of 2-D PAGE gels of the same cultures. Neurons were prepared from: (a-d) E8 ciliary ganglia; (e-h) E8 dorsal root ganglia; and (i-l) E5 purified spinal motoneurons. Culture conditions were: (a,c) 20 ng/ml CNTF; (e,g) 50 ng/ml NGF; (i,k) 10 μg/ml chicken muscle extract; (b,d,f,h,j,l) basal medium. Spots numbered from 1 to 5 on the autoradiograph in d are significantly increased in intensity in pre-apoptotic cCG neurons (d) compared to surviving cCG neurons (c). Spots A and B are spots whose intensity did not change with culture conditions. They were used to quantify the relative levels of Spots 1 to 5 in the different systems. Spots 1 and 2 are circled on autoradiograms from cDRG cultures (g,h) and motoneuron cultures (k,l). Positions of molecular mass markers in the second dimension gel are indicated in d.

cultured in the presence or absence of dexamethasone with a radiolabeled extract from cCG neurons and performed 2-D PAGE. As with rat DRG neurons, levels of the two actin fragments were significantly increased (data not shown).

To quantify relative increases in intensity of actin fragments in cells undergoing apoptosis, amounts of Spot 1 in the three types of chicken neurons (Fig. 1) and in the rat neurons (Fig. 3a) were determined by densitometric scanning of autoradiograms. A representative example of such measures is presented in Fig. 3c, as the ratio of the intensity of spot 1 to the mean intensity of the non-variant internal controls, spots A and B. The relative increase in levels of actin fragments (approximately 5-fold) is very similar in the four cases, although they represent different apoptotic systems. For rat

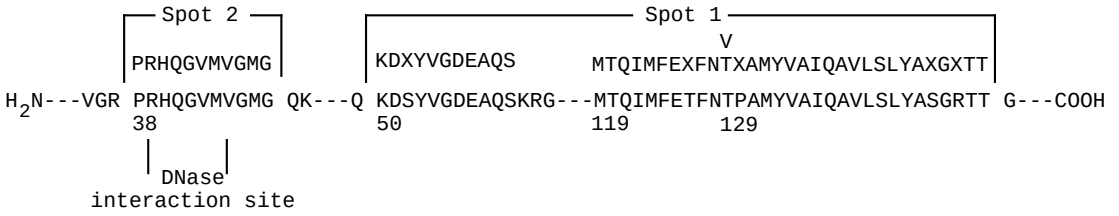
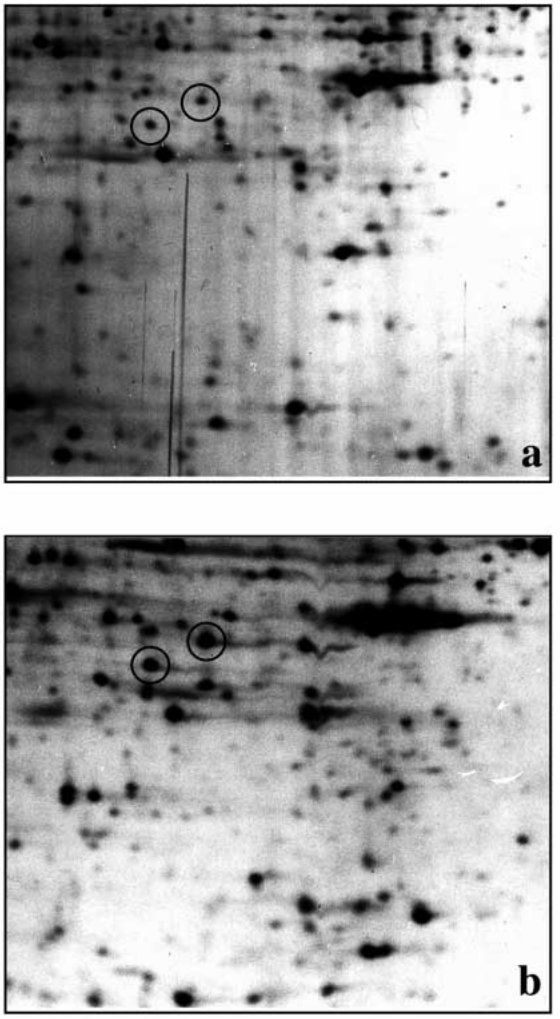


Fig. 2. Sequences of the polypeptides of Spots 1 and 2 showing that they are fragments of actin. Spot 2 was sequenced directly from its NH_2-terminal. However, because Spot 1 needed to be digested after blotting, the 2 internal sequences thus obtained are shown. Residue numbering is that for beta actin; the approximate site of interaction with DNase I is indicated.



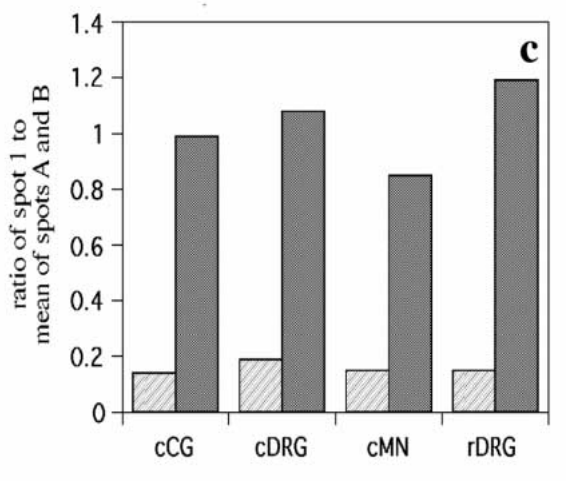


Fig. 3. The same actin fragments accumulate during apoptosis of rat neurons. (a) Silver-stained 2-D PAGE gel of 100 µg protein extracted from unlabeled rat DRG deprived of NGF. Note the strong presence of spots 1 and 2. (b) Autoradiogram of 2 µg protein from radiolabeled chicken CG deprived of CNTF analysed on the same gel. The position of spots 1 and 2 is totally conserved between rat and chicken. (c) Representative results (2 to 4 different experiments depending on the cell type) of a densitometric analysis of the increase in levels of Spot 1, expressed as a ratio between the intensity of spot 1 and the mean intensity of Spots A and B (see Fig. 1d). Hatched bars, neurons cultured in the presence of trophic support. Solid bars, neurons deprived of trophic support.

thymocyte cultures, the relative increase level, which had to be estimated on silver-stained gels, was found to be approximately 3-fold (data not shown).

Actin proteolysis during neuronal apoptosis requires a protease of the calpain family

The presence of the same fragments of actin in four different apoptotic systems in the absence of general proteolysis suggested that they might reflect a common step in the apoptotic cascade. We therefore asked whether in intact cells, actin fragmentation could be prevented by specific protease inhibitors. The two major protease families shown to play a role in the apoptosis of different cell types are the caspases and the calpains. As a first step, we evaluated the survival effect of inhibitors of these two protease families. They were tested on ciliary ganglion neurons that were freshly isolated from E8 embryos, and had not received prior exposure in culture to neurotrophic factors.

For survival measurements, cCG neurons were cultured for 24 hours (a time at which we previously showed that half of the neurons in untreated cultures die) and surviving neurons were counted microscopically after staining with the vital dye MTT. In these conditions about 55% of the neurons died in basal medium, whereas CNTF (20 ng/ml) maintained nearly

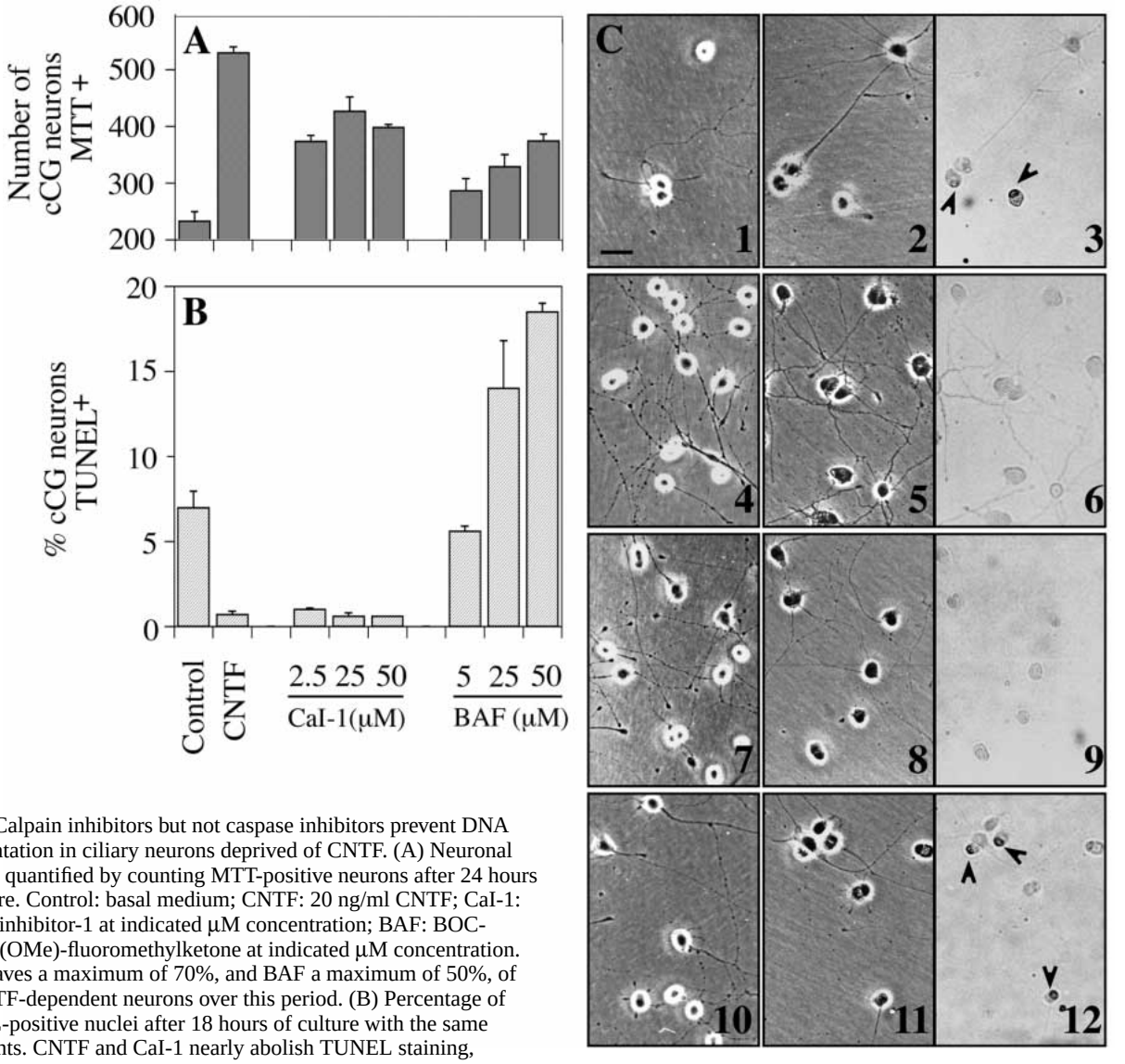


Fig. 4. Calpain inhibitors but not caspase inhibitors prevent DNA fragmentation in ciliary neurons deprived of CNTF. (A) Neuronal survival quantified by counting MTT-positive neurons after 24 hours of culture. Control: basal medium; CNTF: 20 ng/ml CNTF; CaI-1: calpain inhibitor-1 at indicated μM concentration; BAF: BOC-aspartyl(OMe)-fluoromethylketone at indicated μM concentration. CaI-1 saves a maximum of 70%, and BAF a maximum of 50%, of the CNTF-dependent neurons over this period. (B) Percentage of TUNEL-positive nuclei after 18 hours of culture with the same treatments. CNTF and CaI-1 nearly abolish TUNEL staining, whereas BAF-treated neurons show a higher percentage of TUNEL labeling than controls. (C) Morphology of TUNEL-labeled neurons in different culture conditions (bar, 30 μm): (1-3) basal medium; (4-6) 20 ng/ml CNTF; (7-9) 25 μM CaI-1; (10-12) 25 μM BAF. Micrographs are shown of: (1,4,7,10) living cells; (2,5,8,11) fixed cells viewed by phase contrast to show cell morphology; (3,6,9,12) the same fields viewed by transmission to visualize TUNEL-positive nuclei (arrowheads).

100% (Fig. 4A). At concentrations ranging from 2.5 to 50 μM, calpain inhibitor-1 (CaI-1) saved up to 70% of the cCG neurons that died in the absence of CNTF (Fig. 4A). Similar results were obtained with calpain inhibitor-2 (not shown). Both the caspase inhibitors Z-VAD-FMK and BAF prevented death of cCG neurons in the range of 10 to 50 μM (data for BAF shown in Fig. 4A). However, the maximal effect obtained with BAF (50% of the CNTF-responsive neurons) was slightly lower than with CaI-1, and concentrations higher than 50 μM led to decreased survival (data not shown). Thus, apoptosis of fractions of trophic factor-deprived cCG neurons requires the action of members of both calpain and caspase families. The survival effects of the caspase and calpain inhibitors were not found to be additive (data not shown). Therefore, these two protease families probably act, at least partially, at different points along the same pathway.

As a second step, we asked whether either the caspases or the calpains might be involved in the proteolytic cleavage of actin we had described. cCG neurons were therefore cultured for 18 hours and after metabolic labeling, were analysed by 2-D PAGE and autoradiography. As before, actin fragments 1 and 2 were abundant in the absence of CNTF (Fig. 5a) but strongly diminished in its presence (Fig. 5b). When added in the absence of CNTF, CaI-1 (25 μM) led to a diminution of intensity of the same spots relative to the internal control (Fig. 5c). In contrast, BAF (25 μM) did not prevent actin fragmentation (Fig. 5d). Similar results were obtained with Z-VAD-FMK (one experiment, 10 μM, data not shown). As a positive control for the efficacy of our preparations of caspase inhibitors, we checked that they were able to prevent the cleavage of PARP in HeLa cells in which apoptosis was induced by etoposide. At the same concentrations used to study

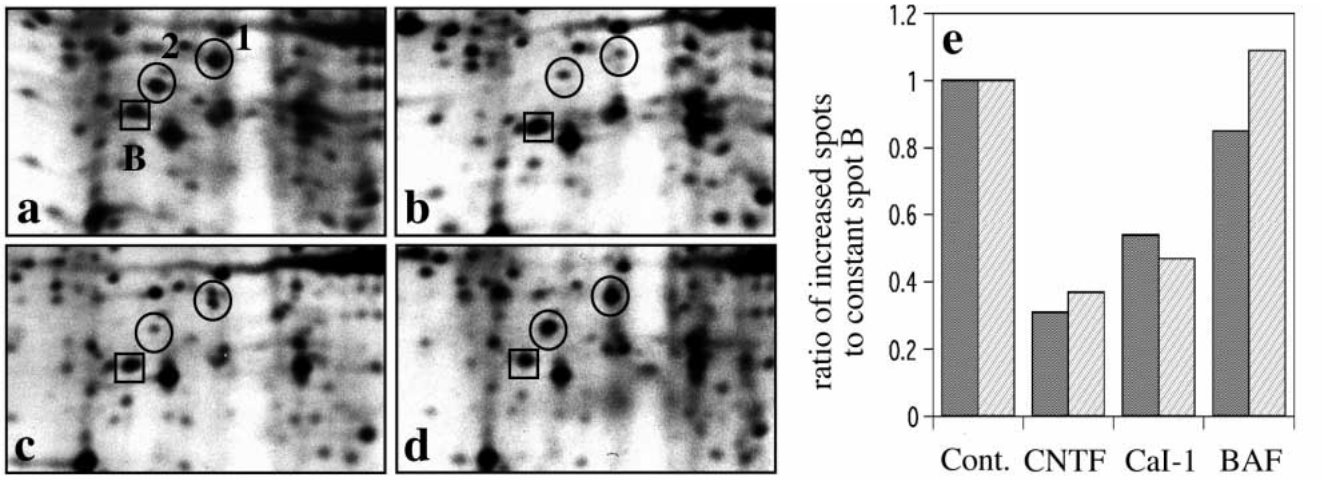


Fig. 5. Calpain inhibitors but not caspase inhibitors are able to inhibit the fragmentation of actin. Autoradiograms of 2-D PAGE gels of cCG neurons cultured for 18 hours in: (a) basal medium; (b) 20 ng/ml CNTF; (c) 25 μM CAI-1, a calpain inhibitor; and (d) 25 μM BAF, a general caspase inhibitor. Spots 1 and 2 are circled on each autoradiogram, and the position of constant Spot B is indicated by a square.
(e) Densitometric quantification (representative data of two different sets of autoradiograms) of the ratio of Spot 1 (solid bars) or Spot 2 (hatched bars) to Spot B.

actin fragmentation, the two inhibitors, ZVAD-FMK (10 μM) and BAF (25 μM), indeed blocked 90% of the cleavage of PARP (data not shown). The effects of the protease inhibitors on actin fragmentation were quantified by densitometric scanning of the autoradiograms; both Spots 1 and 2 were diminished to nearly the same extent by CaI-1 as by CNTF (Fig. 5e), whereas BAF had no effect. We therefore conclude that a calpain-like enzyme is likely to be involved in the in vivo truncation of actin.

Actin proteolysis is correlated with DNA fragmentation

The correlation between the survival effect of the calpain inhibitor-1 and its ability to reduce actin fragmentation raised the question of the role of actin proteolysis in the apoptotic cascade. One possibility would be that it underlies the morphological changes observed during apoptosis. Truncation of actin, together with the truncation of fodrin shown by Martin et al. (1995) could help to reduce the stiffness of the cytoskeleton, allowing the formation of apoptotic bodies without bursting of the cytoplasmic membrane.

A second possible role is based on the property of intact actin to bind to DNase I and inhibit its activity. DNase I was shown by Peitsch et al. (1993) to be the nuclease responsible for the degradation of DNA during the apoptosis of dexamethasone-treated rat thymocytes. These authors also demonstrated that this DNase I activity could be inhibited by actin. The site of cleavage that generates the actin fragment of spot 2, whose N-terminal could be sequenced directly (Fig. 2), corresponds closely to one of the major binding sites on actin for DNase I (Kabsch et al., 1990). Therefore, cleavage of actin could release the DNase I, thereby allowing the now active nuclease to cut the genomic DNA into oligosome-sized fragments. To test this hypothesis, we used TUNEL staining to detect DNA fragmentation in nuclei of cultured cCG neurons (Fig. 4C). When cultured for 24 hours in the absence of CNTF, many of these neurons died (Fig. 4C, panels 1, 2; and Fig. 4A). Of the neurons still present at this stage, many showed positive labelling for TUNEL as expected (Fig. 4C, panels 2, 3); overall, 7% of the surviving neurons were labeled (Fig. 4B). In the presence of CNTF (20 ng/ml), more neurons survived (Fig. 4C, panels 4, 5) and, in keeping with the healthy appearance of the neurons, TUNEL labeling was strikingly reduced to less than 1% (Fig. 4C, panels 5, 6; and Fig. 4B).

At all concentrations tested, CaI-1 almost completely prevented the appearance of TUNEL-positive nuclei over this period (Fig. 4C, panels 7-9; Fig. 4B). Surprisingly, however, the percentage of TUNEL-positive neurons in cultures treated with 25 μM and 50 μM BAF was even higher than in controls (Fig. 4C, panels 11, 12; and Fig. 4B), suggesting that the limited survival effect observed with BAF over the 18-hour test period represented a delay in cell death, rather than a complete protection. In accordance with this, the number of surviving neurons in BAF-treated cultures after 36 hours was not significantly greater than in basal medium, whereas survival in CaI-treated cultures was still about 70% of that in CNTF (not shown). Moreover, a direct toxic effect of BAF could be excluded as the addition of BAF to CNTF-supported neurons had no effect on cell death (not shown). There is thus a strong correlation between inhibition of actin proteolysis, inhibition of DNA fragmentation and neuronal survival.

DISCUSSION

We have shown that levels of two conserved polypeptides increase during apoptosis in different cell types and different species. These polypeptides are specific proteolytic fragments of actin that appear at a stage of apoptosis before massive protein degradation is evident. Their appearance is inhibited by calpain inhibitors, which also prevent the death of many ciliary neurons, and completely prevent nuclear DNA fragmentation. Caspase inhibitors, although they prevent cell death, inhibit neither actin fragmentation nor DNA fragmentation. We propose that actin is an early substrate for calpains during

apoptosis, and that its cleavage is required for subsequent activation of the DNase(s) responsible for DNA fragmentation.

Our approach using 2-D PAGE to analyse the differences in the polypeptide patterns of apoptotic and non-apoptotic cells has several advantages. First, it provides a fairly complete view of the changes in a large number of polypeptides. It was striking that the number of spots whose intensity reproducibly changed during the early stages of apoptosis was low (Villa et al., 1994). Obviously, some changes may be below the levels of sensitivity of autoradiographic detection, but it is likely that those that are visible do indeed represent a specific response to apoptosis. Secondly, it has the advantage over differential and subtractive cDNA approaches of being able to detect post-translational modifications such as the actin fragmentation reported here. Thirdly, it provides a means of observing in a non-invasive manner the changes that occur in cells that are dying in conditions that are probably close to those involved in early development of the nervous system, where competition for limited stocks of neurotrophic factors is thought to induce death of approximately half of the neurons initially produced (Oppenheim, 1991).

It is unlikely that general proteolysis in dying cells can explain our observation of proteolytic fragments of an abundant protein such as actin. The low number of polypeptides present at increased levels after 18 hours of trophic deprivation indicates that general proteolysis has not begun at this stage of the apoptotic process (Villa et al., 1994). Indeed, even when gels were overloaded so as to allow detection of at least 2500 spots in the pI 5-8 range with molecular mass between 20 and 100 kDa, no further significant differences were observed (not shown). In addition, although no detailed time-course study was done, we observed the presence of the fragments already at 12 hours, a time at which there is clearly no cell death and therefore no general proteolysis yet (not shown). Finally, in apoptotic 3T3 fibroblasts, while Gas-2, a low-abundance protein, is processed from a 35 kDa to a 31 kDa form, major proteins such as tubulin and vimentin are not subject to proteolytic degradation, suggesting that the process is specific (Brancolini et al., 1995).

This suggested that the fragmentation of actin we observed involved the action of a specific proteolytic enzyme, and perhaps one of those that have been shown to be major actors in the induction of cell death. We concentrated our attention on the calpains and the caspases, because of their demonstrated role in neuronal apoptosis, and because there are selective non-toxic inhibitors available for each class.

That actin may be a substrate for one of these proteases was first suggested in vitro by Mashima et al. (1995, 1997). Another study published by Kayalar et al., (1996) showed that when extracts of PC12 cells are treated with caspase-1, actin can be cut first into a 41 kDa fragment, then into two pieces of 30 and 14 kDa. These actin fragments were unable to bind DNase I and inhibit its enzymatic activity. In the same study, it was reported that in apoptotic serum-deprived PC12 cells, a different actin fragment (30 kDa) from that obtained in vitro appeared, demonstrating that experiments in which cell lysates are used as substrates of proteases are not directly extrapolable to whole cells. Other groups (MacCarthy et al., 1997; Brown et al., 1997) confirmed that actin could partially be cleaved by caspases. In contrast, Polverino and Patterson (1997) and Song et al. (1997) claimed that actin is resistant to cleavage during apoptosis. However, actin fragments may not be resolved in the one-dimensional gels used and these authors used antibodies to epitopes that may be absent from actin after cleavage. Only one study was performed on primary cells (Brown et al., 1997) and reported that, in neutrophils undergoing apoptosis, actin is cleaved by a calpain-like enzyme after residue Val 43 (β actin).

We show here that actin cleavage after an Arg residue (37 in β actin/39 in α actin) is a general feature of neuronal and non-neuronal cells that have just entered the apoptotic programme. Our results suggest that during apoptosis, actin is likely to be cut first by a calpain-like enzyme, although the further action of a caspase family member cannot be excluded. In support of our results excluding the action of a caspase, the site of cleavage (between Arg and Pro residues) which generates the NH_2-terminal of the actin fragment of Spot 2 is not in accordance with the specificity of proteases of the caspase family, which cut after an Asp residue (Sleath et al., 1990). The specificity of calpains seems to be much broader, although substrates have been shown to be cut only at one site in most cases. Studies from Stabach et al. (1997) show that the secondary and tertiary conformation of the substrate are more important for the calpain than the primary sequence. For example, fodrin, a high molecular mass protein (240 kDa), shows only a single 120 kDa polypeptide fragment when cleaved by a calpain (Martin et al., 1995).

What might be the role(s) of the actin fragmentation in the process of apoptosis? One possibility is that it underlies the morphological changes necessary for the formation of apoptotic bodies. This would place this event, like the fragmentation of spectrin or fodrin (Martin et al., 1995), at the end of the apoptotic process, as a consequence of death induction.

Another more specific role is suggested by the fact that actin proteolysis in vivo occurs exactly at the site of interaction between actin and DNase I (Fig. 2). Actin has long been known to bind, with an affinity of 2×10^{-8} M (Polzar et al., 1989), DNase I and inhibit its nuclease activity (Lazarides and Lindberg, 1974). However, although DNase I has often been used to 'decorate' actin microfilaments for electron microscopy (Zimmer and Goldstein, 1987), the physiological explanation for this association remains unclear. As demonstrated by the crystal structure of the complex (Kabsch et al., 1990), binding of actin to DNase I involves strong interactions between the region Arg-39/Val-45 in β actin and the region Asp-53/Val-67 in DNase I. We show here that actin is cut in apoptotic cells just after Arg-37; this cleavage may therefore both release DNase I from its binding site and relieve the inhibition by actin.

It is clear from the examination of the 2-D gels that the percentage of total actin fragmented is very low. In two subfractionation studies, we observed that the fragmented actin was exclusively located in the nuclear fraction (Villa et al., 1994), which may explain why only a fraction of actin is cleaved. The nuclear fraction may have been contaminated with some cytoplasmic cytoskeleton. However, if cytoplasmic actin fragmentation were quantitatively important, then fragments of actin should have been found in the cytoplasmic fraction. We therefore believe that actin is cleaved either near the nucleus or in the nucleus, which is in accordance with the idea that this truncation of actin is involved in DNase activation. Although the available data show all an interaction

between actin and DNase I, we cannot exclude the possibility that other DNases could play a similar function, such as the DNase gamma described by Shiokawa et al. (1997) to be involved in thymocyte apoptosis, or the DNase II described by Torriglia et al. (1995) which is involved in the apoptosis occuring during lens differentiation.

Actin proteolysis is strongly inhibited by both calpain inhibitors 1 and 2, but not by either BAF or Z-VAD-FMK, two relatively non-specific inhibitors of caspases (Deshmukh et al., 1996; Villa et al., 1997). This suggests that CaI-1 and -2 were acting specifically to inhibit calpain(s), although we cannot completely exclude the possibility that they may also have affected proteasome activity (Sadoul et al., 1996; Drexler, 1997). We could not demonstrate involvement of caspases in actin fragmentation, but cannot exclude a possible downstream role in actin degradation.

Both calpains and caspase members seem to be involved in apoptosis of ciliary neurons, since inhibitors of each could considerably retard cell death in CNTF-deprived cultures (Fig. 4A). It was striking, however, that even at optimal concentrations neither inhibitor could completely prevent cell death. This is in contrast to results obtained using sympathetic neurons cultured without NGF in the presence of either caspase inhibitors (Deshmukh et al., 1996) or calpain inhibitors (Sadoul et al., 1996). The explanation for this may lie either in the fact that the ciliary ganglion contains two classes of neuron (ciliary and choroid) (Marwitt et al., 1971), and/or in the fact that different ciliary neurons have received prior exposure to different neurotrophic factors (Allsopp et al., 1993). Death of the same neurons induced by different signals may involve different proteases (Troy et al., 1996a,b).

There was a strong correlation in our experiments between actin fragmentation and DNA fragmentation; both were inhibited by calpain inhibitors over a period of 18 hours but neither were affected by caspase inhibitors. Nevertheless, both sets of inhibitors prevented cell death to a similar degree for 24 hours in vitro. This suggests that, for a certain period at least, actin and DNA fragmentation may both be uncoupled from morphological cell death (see the TUNEL-positive neurons in the presence of BAF in Fig. 4C). This is in agreement with the report that apoptosis may occur in cells in the absence of a nucleus (Jacobson et al., 1994). However, the two processes of fragmentation (of actin and DNA) seem to be linked, suggesting that DNA fragmentation may require actin proteolysis, and that release of DNase I by the action of calpain may play an important role in apoptosis. Conclusive proof of the hypothesis would require mutation of the putative calpain cleavage site after Arg-37; however, interpretation of the results would be complicated by the probable interference of such mutations with actin-DNase I interactions.

Protease action during apoptosis is likely to be complex and varied (Martin and Green, 1995; Takahashi and Earnshaw, 1996). Although many proteolytic events are undoubtedly required to produce the characteristic morphological changes associated with apoptosis, those reported here may be of particular interest. Actin degradation at its site of interaction with DNase I occurs at a stage of apoptosis before massive proteolysis can be detected, and seems to affect a specific pathway leading to DNA degradation, that for a certain period may occur independently of morphological cell death.

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REFERENCES

Allsopp, T. E., Wyatt, S., Paterson, H. F. and Davies, A. M. (1993). The proto-oncogene bcl-2 can selectively rescue neurotrophic factor-dependent neurons from apoptosis. *Cell* **73**, 295-307.

Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong W. W. andYuan, J. (1996). Human ICE/CED-3 protease nomenclature. *Cell* **87**, 171.

Bloch-Gallego, E., Huchet, M., El M'HamdiS, H., Xie, F. K., Tanaka, H. and Henderson, C. E. (1991). Survival in vitro of motoneurons identified or purified by novel antibody-based methods is selectively enhanced by muscle-derived factors. *Development* **111**, 221-232.

Brancolini, C., Benedetti, M. and Schneider, C. (1995). Microfilament reorganization during apoptosis: the role of Gas2, a possible substrate for ICE-like proteases. *EMBO J.* **14**, 5179-5190.

Brown, S. B., Bailey K. and Savill, J. (1997). Actin is cleaved during constitutive apoptosis. *Biochem. J.* **323**, 233-237.

Camu, W., Bloch-Gallego, E. and Henderson, C. E. (1993). Purification of spinal motoneurons from chicken and rat embryos by immunopanning. *Immunoselection Strategies for Neural Cell Culture, Neuroprotocols: A Companion to Methods in Neurosciences.* **2**, 191-199.

Casciola-Rosen, L. A., Anhalt, G. J. and Rosen, A. (1995). DNA-dependant protein kinase is one of the subset of autoantigens specifically cleaved early during apoptosis. *J. Exp. Med.* **182**, 1625-1634.

Cowan, W. M., Fawcett, J. W., O'Leary, D. D. and Stanfield B. B. (1984). Regressive events in neurogenesis. *Science* **225**, 1258-1265.

Deshmukh, M., Vasilakos, J., Deckwerth, T. L., Lampe, P. A., Shivers, B. D. and Johnson, E. M. Jr (1996). Genetic and metabolic status of NGF-deprived sympathetic neurons saved by an inhibitor of ICE family proteases. *J. Cell Biol.* **135**, 1341-1354.

Drexler, H. C. (1997). Activation of the cell death program by inhibition of proteasome function. *Proc. Nat. Acad. Sci. USA* **94**, 855-860.

Ellis, H. M. and Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode C. elegans. *Cell* **44**, 817-829.

Ellis, R. E., Yuan, J. Y. and Horvitz, H. R. (1991). Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.* **7**, 663-698.

Garrels, J. I. (1979). Two dimensional gel electrophoresis and computer analysis of proteins synthesized by clonal cell lines. *J. Biol. Chem.* **254**, 7961-7977.

Gu, Y., Sarnecki, C., Aldape, R. A., Livingston, D. J. and Su, M. S. (1995). Cleavage of poly(ADP-ribose) polymerase by interleukin-1 beta converting enzyme and its homologs TX and Nedd-2. *J. Biol. Chem.* **270**, 18715-18718.

Henderson, C. E., Bloch-Gallego, E. and Camu, W. (1995). Purified embryonic motoneurons. In *Nerve Cell Culture: a Practical Approach* (ed. J. Cohen and G. Wilkin). pp. 69-81. Oxford University Press, London.

Henderson, C. E. (1996). Programmed cell death in the developing nervous system. *Neuron* **17**, 579-585.

Hengartner, M. O., Ellis, R. E. and Horvitz, H. R. (1992). Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. *Nature* **356**, 494-499.

Henzel, W. J., Grimley, C., Bourrel, J. H., Billeci, T. M., Wong, S. C. and Stults, J. T. (1994). Analysis of two-dimensional gel proteins by mass spectrometry and microsequencing. *Methods: A Companion to Methods in Enzymology* **6**, 239-247.

Humblet, C., Deman, J., Franzen, R., Greimers, R., Boniver, J. and Defresne, M. P. (1995). Spontaneous and induced apoptosis after whole body radiation exposure: experimental approaches. Observations in radio-induced thymic lymphomagenesis. *Stem Cells* **1**, 129-135.

Jacobson, M. D., Burne, J. F. and Raff, M. C. (1994. Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO J.* **13**, 1899-1910.

Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F. and Holmes, K. C. (1990). Atomic structure of the actin:DNase I complex [see comments]. *Nature* **347**, 37-44.

Kayalar, C., Ord, T., Testa, M. P., Zhong, L. T. and Bredesen, D. E. (1996).

Cleavage of actin by interleukin 1 beta-converting enzyme to reverse DNase I inhibition. *Proc. Nat. Acad. Sci. USA* **93**, 2234-2238.

Kuida, K., Lippke, J. A., Ku, G., Harding, M. W., Livingston, D. J., Su, M. and Flavell, R. A. (1995). Altered cytokine export and apoptosis in mice deficient in interleukin-1-beta converting-enzyme. *Science* **267**, 2000-2003.

Kuida, K., Zheng, T. S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P. and Flavell, R. A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* **384**, 368-372.

Lazarides, E. and Lindberg, U. (1974). Actin is the naturally occurring inhibitor of deoxyribonuclease I. *Proc. Nat. Acad. Sci. USA* **71**, 4742-4746.

Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G. and Earnshaw, W. C. (1994). Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371**, 346-347.

Lazebnik, Y. A., Takahashi, A., Moir, R. D., Goldman, R. D., Poirier, G. G., Kaufmann, S. H. and Earnshaw, W. C. (1995). Studies of the lamin proteinase reveal multiple parallel biochemical pathways during apoptotic execution. *Proc. Nat. Acad. Sci. USA* **92**, 9042-906.

Levi-Montalcini, R. (1964). Events in the developing nervous system. *Prog. Brain Res.* **4**, 1-29.

Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., Mcdowell, J., Paskind, M., Rodman, L., Salfeld, J. Towne, E., Tracey, D. Wardwell, S., Wei, F. Y., Wong, W., Kamen, R. and Seshadri, T. (1995). Mice deficient in IL-1-beta-converting enzyme are defective in production of mature IL-1-beta and resistant to endotoxic-shock. *Cell* **80**, 401-411.

Loret, C., Sensenbrenner, M. and Labourdette, G. (1988). Maturation-related gene expression of rat astroblasts in vitro studied by two-dimensional polyacrylamide gel electrophoresis. *Cell Differ Dev*. **25**, 37-45.

MacCarthy, N. J., Whyte, M. K. B., Gilbert C. S. and Evan, G. (1997). Inhibition of Ced-3/ICE proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. *J. Cell Biol.* **136**, 215-227.

Manthorpe, M., Fagnani, R., Skaper, S. D. and Varon, S. (1986). An automated colorimetric microassay for neuronotrophic factors. *Brain Res*. **390**, 191-198.

Martin, S. J. and Green, D. R. (1995). Protease activation during apoptosis: death by a thousand cuts? *Cell* **82**, 349-352.

Martin, S. J., O'Brien, G. A., Nishioka, W. K., McGahon, A. J., Mahboubi, A., Saido T. C. and Green, D. R. (1995). Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. *J. Biol. Chem*. **270**, 6425-6428.

Martins, L. M. and Earnshaw, W. C. (1997). Apoptosis: alive and kicking in 1997. *Trends Cell Biol.* **7**, 11-14.

Marwitt, R., Pilar, G. and Weakly, J. N. (1971). Characterization of two ganglion cell populations in avian ciliary ganglia. *Brain Res*. **25**, 317-334.

Mashima, T., Naito, M., Fujita, N., Noguchi, K. and Tsuruo, T. (1995). Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in VP-16-induced U937 apoptosis. *Biochem. Biophys. Res. Commun*. **217**, 1185-1192.

Mashima, T., Naito, M., Noguchi, K., Miller, D. K., Nicholson, D. W. and Tsuruo, T. (1997). Actin cleavage by CPP-32/apopain during the development of apoptosis. *Oncogene* **14**, 1007-1012.

Merril, C. R., Goldman, D., Sedman, S. A. and Ebert, M. H. (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211**, 1437-1438.

Milankov, K. and De, B. U. (1993) Cytochemical localization of actin and myosin aggregates in interphase nuclei in situ. *Exp. Cell Res*. **209**, 189-199.

Milligan, C. E., Prevette, D., Yaginuma, H., Homma, S., Cardwell, C., Fritz, L. C., Tomaselli, K. J., Oppenheim, R. W. and Schwartz, L. M. (1995). Peptide inhibitors of the ICE protease family arrest programmed cell death of motoneurons in vivo and in vitro. *Neuron* **15**, 385-393.

Miura, M., Zhu, H., Rotello, R., Hartwieg, E. A. and Yuan, J. (1993). Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. *Cell* **75**, 653-660.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem*. **250**, 4007-4021.

Oppenheim, R. W. (1985). Naturally occurring cell death during neural development. *Trends Neurosci.* **8**, 487-493.

Oppenheim, R. W. (1991). Cell death during development of the nervous system. *Annu. Rev. Neurosci.* **14**, 453-501.

Peitsch, M. C., Polzar, B., Stephan, H., Crompton, T., MacDonald, H. R., Mannherz, H. G. and Tschopp, J. (1993). Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). *EMBO J*. **12**, 371-377.

Polverino, A. J. and Patterson, S. D. (1997). Selective activation of caspases during apoptotic induction in HL-60 cells. *J. Biol. Chem.* **272**, 7013-7021.

Polzar, B., Nowak, E., Goody, R. S. and Mannherz, H. G. (1989). The complex of actin and deoxyribonuclease I as a model system to study the interactions of nucleotides, cations and cytochalasin D with monomeric actin. *Eur. J. Biochem*. **182**, 267-275.

Roberts-Lewis, J. M., Savage, M. J., Marcy, V. R., Pinsker, L. R. and Siman, R. (1994). Immunolocalization of calpain I-mediated spectrin degradation to vulnerable neurons in the ischemic gerbil brain. *J. Neurosci*. **14**, 3934-3944.

Sadoul, R., Fernandez, P. A., Quiquerez, A. L., Martinou, I., Malki, M., Schroter, M., Becherer, J. D., Irmler, M., Tschopp, J. and Martinou, J. C. (1996). Involvement of the proteasome in the programmed cell death of NGF-deprived sympathetic neurons. *EMBO J*. **15**, 3845-3852.

Shiokawa, D., Ohyama, H., Yamada, T. and Tanuma, Si. (1997). Purification and properties of DNase gamma from apoptotic rat thymocytes. *Biochem. J.* **326**, 675-681.

Sleath, P. R., Hendrickson, R. C., Kronheim, S. R., March, C. J. and Black, R. A. (1990). Substrate specificity of the protease that processes human interleukin-1 beta. *J. Biol. Chem*. **265**, 14526-14528.

Song, Q., Lees-Miller, S. P., Kumar, S., Zhang, Z., Chan, D. W., Smith, G. C., Jackson, S. P., Alnemri, E. S., Litwack, G., Khanna, K. K. and Lavin, M. F. (1996). DNA-dependent protein kinase catalytic subunit: a target for an ICE-like protease in apoptosis. *EMBO J*. **15**, 3238-3246.

Song, Q., Wei, T., Lees-Miler, S., Alnemri, E., Watters, D. and Lavin, M. F. (1997). Resistance of actin to cleavage during apoptosis. *Proc. Nat. Acad. Sci. USA* **94**, 157-162.

Squier, M. K., Miller, A. C., Malkinson, A. M. and Cohen, J. J. (1994). Calpain activation in apoptosis. *J. Cell. Physiol.* **159**, 229-237.

Stabach, P. R., Cianci, C. D., Glantz, S. B., Zhang, Z. and Morrow, J. S. (1997). Site-directed mutagenesis of alpha II spectrin at codon 1175 modulates its mu-calpain susceptibility. *Biochemistry* **36**, 57-65.

Takahashi, A. and Earnshaw, W. C. (1996). ICE-related proteases in apoptosis. *Curr. Opin. Genet. Dev.* **6**, 50-55.

Torriglia, A., Chaudun, E., Chanyfournier, F., Jeanny, J. C., Courtois, Y. and Counis, M. F. (1995) Involvement of DNase II in nuclear degeneration during lens cell differentiation. *J. Biol. Chem.* **270**, 28579-28585.

Troy, C. M., Derossi, D., Prochiantz, A., Greene, L. A. and Shelanski, M. L. (1996a). Downregulation of Cu/Zn superoxide dismutase leads to cell death via the nitric oxide-peroxynitrite pathway. *J. Neurosci.* **16**, 253-261.

Troy, C. M., Stefanis, L., Prochiantz, A., Greene, L. A. and Shelanski, M. L. (1996b). The contrasting roles of ICE family proteases and interleukin-1beta in apoptosis induced by trophic factor withdrawal and by copper/zinc superoxide dismutase down-regulation. *Proc. Nat. Acad. Sci. USA* **93**, 5635-5640.

Vandekerckhove, J. and Weber, K. (1979). The complete amino-acid sequence of actins from bovine aorta, bovine heart, bovine fast skeletal muscle and rabbit slow skeletal muscle. *Differentiation* **14**, 123-133.

Villa, P., Miehe, M., Sensenbrenner, M. and Pettmann, B. (1994). Synthesis of specific proteins in trophic factor-deprived neurons undergoing apoptosis. *J. Neurochem.* **62**, 1468-1475.

Villa, P., Kaufmann, S. H. and Earnshaw, W. C. (1997). Caspases and caspase inhibitors. *Trends Biochem. Sci.* **22**, 388-393.

Zimmer, D. B. and Goldstein, M. A. (1987). DNase I interactions with filaments of skeletal muscles. *J. Muscle Res. Cell Motil*. **8**, 30-38.