J. Biochem. 130, 535–542 (2001)

Limited Proteolysis of Filamin Is Catalyzed by Caspase-3 in U937 and Jurkat Cells[1]

Tatsuya Umeda,* Zen Kouchi,* Hiroyuki Kawahara,* Shigeo Tomioka,* Noboru Sasagawa,† Tatsuya Maeda,* Hiroyuki Sorimachi,* Shoichi Ishiura,†,[2] and Koichi Suzuki*

*Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032; and †Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan

Received May 28, 2001; accepted August 2, 2001

Members of the caspase family have been implicated as key mediators of apoptosis in mammalian cells. However, few of their substrates are known to have physiological significance in the apoptotic process. We focused our screening for caspase substrates on cytoskeletal proteins. We found that an actin binding protein, filamin, was cleaved from 280 kDa to 170, 150, and 120 kDa major N-terminal fragments, and 135, 120, and 110 kDa major C-terminal fragments when apoptosis was induced by etoposide in both the human monoblastic leukemia cell line U937, and the human T lymphoblastic cell line Jurkat. The cleavage of filamin was blocked by a cell permeable inhibitor of caspase-3–like protease, Ac-DEVD-cho, but not by an inhibitor of caspase-1–like protease, Ac-YVAD-cho. These results suggest that filamin is cleaved by a caspase-3–like protease. To examine whether caspase-3 cleaves filamin *in vitro*, we prepared a recombinant active form of caspase-3 directly using a *Pichia pastoris* overexpression system. When we applied recombinant active caspase-3 to the cell lysate of U937 and Jurkat cells, filamin was cleaved into the same fragments seen in apoptosis-induced cells *in vivo*. Platelet filamin was also cleaved directly from 280 kDa to 170, 150, and 120 kDa N-terminal fragments, and the cleavage pattern was the same as observed in apoptotic human cells *in vivo*. These results suggest that filamin is an *in vivo* substrate of caspase-3.

Key words: apoptosis, caspase-3, cytoskeleton, filamin, proteolysis.

Apoptosis is a system of eliminating unnecessary cells and essential for development, organization, and homeostasis. It is concerned with the removal of unnecessary cells during processes such as immune selection, establishment of the neural network, and inflammation. Therefore, apoptosis comprises a series of processes causing DNA fragmentations and morphological changes such as chromatin condensation, microvilli disappearance, shrinkage, and the generation of apoptotic bodies, including the phagocytotic removal of damaged cells (*1–6*).

Molecular research on apoptosis has been making rapid progress since many genes involved in cell death in *Caenorhabditis elegans* have been identified. A mammalian homolog of the cell death execution factors found in *C. elegans* has been isolated, and its biochemical characterization has progressed, resulting in its general acceptance among cell death molecules (*7, 8*). Since the discovery that Ced-3, a key regulatory gene of cell death in *C. elegans*, exhibits homology with interleukin-1β converting enzyme (ICE) (*9*), several mammalian ICE/Ced-3 homologues, together called the caspase family, aspartate-specific proteases comprising 14 members in mammals, have been identified (*10–27*). Concerning the morphological changes that occur during apoptosis, it has been reported that some important structural proteins, such as lamin (*28*), α-fodrin (*29, 30*), and actin (*31*), undergo limited proteolysis in apoptotic cells. These cytoskeletal proteins are well-known substrates for caspases. Some of these cleavage events are considered to be important for apoptotic morphological changes, but it remains to be learned whether the proteolysis of other cytoskeletal proteins occurs, and whether this has a significant effect on the apoptotic changes.

Using the human monoblastic leukemia cell line U937, and the human T lymphoblastic cell line Jurkat, we focused on screening for caspase substrates on cytoskeletal proteins, and tried to elucidate part of an unknown regulatory mechanism for the morphological changes. Here, we show that filamin is a substrate for caspase. It has recently been demonstrated by Browne *et al.* that filamin is cleaved in a caspase-dependent manner during Fas mediated apoptosis of Jurkat cells (*32*). We confirmed that filamin is cleaved during apoptosis induced by various stimuli including etoposide, camptothecin, Ara-C, cycloheximide, and H_2O_2. Furthermore, we analyzed the detailed cleavage pattern of filamin by caspase, and used a biochemical approach to demonstrate that this limited proteolysis is catalyzed directly by caspase-3.

[1] This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

[2] To whom correspondence should be addressed. Tel/Fax: +81-3-5454-6739, E-mail: cishiura@mail.ecc.u-tokyo.ac.jp

Abbreviations: Ara-C, cytosine β-D-arabinofuranoside; CHX, cycloheximide; ICE, interleukin-1β–converting enzyme; Ac-YVAD-cho, acetyl-Tyr-Val-Ala-Asp aldehyde; Ac-DEVD-cho, acetyl-Asp-Glu-Val-Asp aldehyde; Ac-YVAD-MCA, acetyl-Tyr-Val-Ala-Asp methylcoumarinamide; Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp methylcoumarinamide; AMC, 7-amino-4-methylcoumarin.

MATERIALS AND METHODS

Induction of Apoptosis—Human U937 myeloid leukemia cells and human Jurkat cells were grown in RPMI1640 medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL). Exponentially growing U937 cells and Jurkat cells were resuspended in RPMI1640 medium with 10% FBS at 5×10^5 cells/ml. For time-course analyses, the cells were treated with 10 μg/ml etoposide (Sigma), 10 μg/ml camptothecin (Sigma), or 10 μg/ml Ara-C (Sigma) for 0, 2, 4, 6, 8, or 24 h, or 10 μg/ml cycloheximide (Sigma), or 10 μM H_2O_2 (Wako) for 0, 12, or 24 h. For protease inhibitor analyses, U937 cells were pretreated for 1 h with the appropriate concentration of Ac-YVAD-cho or Ac-DEVD-cho (the Peptide Institute) and then treated for an additional 8 h with 10 μg/ml etoposide.

Detection and Analysis of Internucleosomal DNA Fragmentation—Cells (5×10^5) were washed with PBS and incubated in 50 μl of 50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsarcosinate (Sigma), and 0.5 μg/ml RNase A (Sigma) at 50°C for 60 min. Five microliters of 0.5 μg/ml proteinase K (Sigma) were added and the cells were incubated for an additional 90 min. The DNA was separated in gels containing 2% agarose/TBE (89 mM Tris-borate, 2 mM EDTA, pH 8.0) buffer at 100 V for 50 min (*33*).

Measurement of Caspase-1 or Caspase-3–Like Protease Activity—U937 and Jurkat cells (1×10^6) treated with etoposide were suspended in 100 μl extraction buffer (50 mM Tris-HCl, pH 7.5, 10 mM β-ME, 1 mM EDTA) and sonicated with a SONIFIER 450 (BRANSON). Ten microliters of cell lysates were then diluted into 100 μl of assay buffer (25 mM Tris-HCl, pH 7.5, 10 mM β-ME, 1 mM EDTA), plus 10 μM Ac-YVAD-MCA or Ac-DEVD-MCA (the Peptide Institute). After incubation at 37°C for 30 min, the change in fluorescence due to the liberation of AMC (the Peptide Institute) was monitored at 460 nm at an excitation wavelength of 380 nm using an FP-777 Spectrofluorometer (JASCO). Standard curves were prepared using solutions of AMC in assay buffer.

Immunoblot Analysis—Proteins were subjected to 7% SDS–polyacrylamide gel electrophoresis and then transferred to PVDF membranes (EIDO). The transfer buffer contained 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 0.037% (w/v) SDS, and 20% (v/v) methanol. The membranes were blocked with 5% skim milk in PBS containing 0.1% Tween 20 (PBS/Tween20). The membranes were then washed three times with PBS/Tween20, and incubated with anti-filamin monoclonal antibodies MAb PM6/317 (Serotec) or MAbTl10 (Chemicon International). The membranes were again washed three times with PBS/Tween20, and then incubated with horseradish peroxidase-conjugated anti-mouse IgG (Vectastain). The antibody complexes were visualized using peroxidase substrate (POD™ immunostaining set, Wako). Quantitative analysis was carried out with Image Master 1D Elite Prime Ver. 2.01 (Amersham Pharmacia Biotech.).

Preparation of Recombinant Caspase-3—Hexahistidine-tagged human caspase-3 was produced in methylotropic yeast, *Pichia pastoris*, and expression was induced in YP medium containing 0.5% (v/v) methanol as described previously (*32*). The His-tagged recombinant protein was purified on a nickel affinity column (QIAGEN) according to the instruction manual.

Preparation of Cytosolic Extracts from U937 and Jurkat Cells—U937 and Jurkat cells were stored at –80°C until used. Cells were lysed by sonication in ice-cold extraction buffer as described above. Extracts were prepared from aliquots of 2×10^5 cells and contained approximately 20 μg of total protein.

Preparation of Human Platelet Filamin—Human platelets were kindly provided by Drs. Masaru Shimizu and Koji Osada, Tokyo Women's University School of Medicine. Platelets were stored at –80°C until used. Platelets were washed twice with H_2O containing 1 mM PMSF (Sigma) as described previously (*34*). Platelets were lysed by sonication in ice-cold buffer as described above. Each extract was prepared from an aliquot of 2×10^6 cells and contained approximately 10 μg of total protein.

RESULTS

Treatment of U937 and Jurkat cells with etoposide induced apoptosis. We assessed nucleosomal fragmentation in apoptotic cells and measured caspase-1 and caspase-3–like protease activities using the synthetic substrates Ac-YVAD-MCA and Ac-DEVD-MCA, respectively. DNA fragmentation occurred within 4 h, and as it proceeded, a remarkable increase in caspase-3–like activity, but not caspase-1–like activity, was detected using the fluoregenic substrates (Fig. 1, A and B). The proteolytic cleavage of caspase-3 was also analyzed by Western blot analysis employing an antibody specific for caspase-3, and the cleavage of the 32 kDa procaspase-3 into a 12 kDa small subunit was confirmed (data not shown). Apoptotic bodies were detected within 4 h after treatment with etoposide (Fig. 1C).

Using this system, we focused on screening for apoptotic substrates on cytoskeletal proteins in floating cells, because a series of morphological changes is a hallmark of apoptosis. Immunoblotting analyses with antibodies to α-actinin and vinculin revealed no obvious changes in the state of these proteins (Fig. 2C). On the other hand, α-spectrin, and α-fodrin were cleaved within 4 h after etoposide treatment as described (*35, 36*).

Importantly, we found that filamin, a major component of actin filaments, was cleaved into 170, 150, and 120 kDa fragments during apoptosis when the blots were probed with an anti-filamin antibody, MAb PM6/317, against the 190 kDa N-terminal fragments catalyzed by calpain (Fig. 2A), and 135, 120, and 110 kDa fragments when the blots were probed with MAb Tl10 against the 90 kDa C-terminal fragments catalyzed by calpain (Fig. 2B). The time course of cleavage of these cytoskeletal proteins is consistent with the appearance of DNA fragmentation and the morphological changes characteristic of apoptosis.

Similarly, U937 cells treated with campthotecin, Ara-C, cycloheximide, or H_2O_2 also underwent rapid apoptosis, and the cleavage of filamin into 170, 150, and 120 kDa N-terminal fragments correlated well with the morphological changes seen in the case of etoposide-induced apoptosis (Fig. 3).

Filamin is a good substrate for calpain *in vitro*, and smooth muscle and platelet filamin have been shown to be cleaved by calpain (*37–39*). However, the cleavage pattern of filamin that accompanies cell death is obviously different

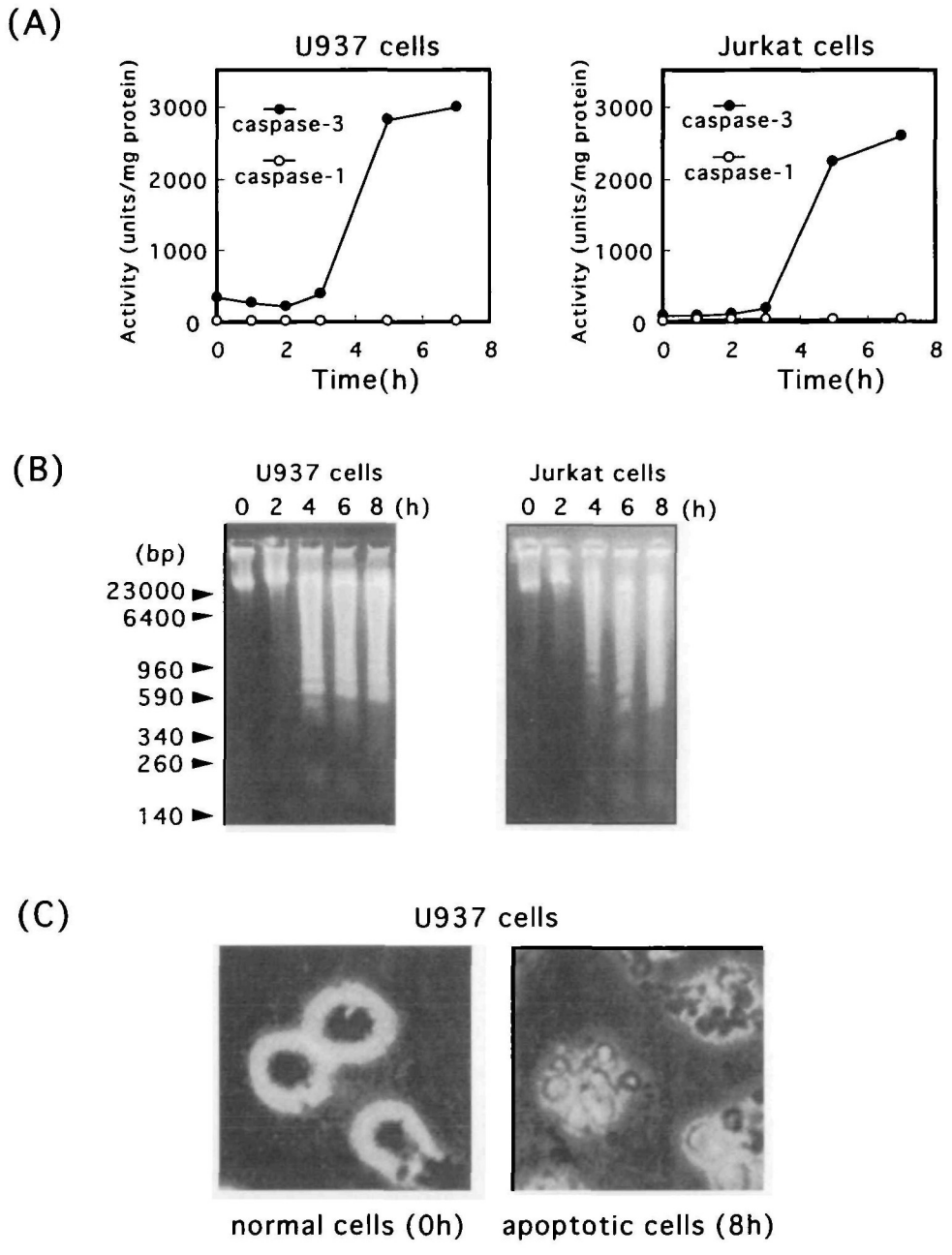

Fig. 1. **Etoposide-mediated apoptosis in U937 and Jurkat cells.** Caspase-1 and caspase-3–like protease activities were measured as described in "MATERIALS AND METHODS" (one unit corresponds to the activity that cleaves 1 pmol of the respective fluorescent substrate at 37°C in 1 min) (A). DNA fragmentation was analyzed by 2% agarose gel electrophoresis. Molecular mass standards are indicated by the arrowheads on the left (B). U937 cells were examined by phase microscopy (C).

from that catalyzed by calpain. The common appearance of proteolyzed fragments in various systems suggests that this cleavage is specific to apoptotic events.

Regarding the progression of apoptosis, it is known that caspase, a cysteine protease, is activated and cleaves nuclear and cytosolic proteins. We focused on caspase, and examined the ability of caspase inhibitors to block the etoposide-induced cleavage of filamin using anti-filamin antibodies MAb PM6/317 (Fig. 4A) and MAb Tl10 (Fig. 4B). U937 cells were preincubated for 1 h with the caspase-1–like protease inhibitor Ac-YVAD-cho, or caspase-3–like protease inhibitor Ac-DEVD-cho prior to the addition of etoposide. Filamin cleavage was only slightly inhibited by Ac-YVAD-cho, but the generation of all fragments was strongly inhibited by Ac-DEVD-cho. Ac-DEVD-cho inhibited cleavage with a concentration dependence over 1 μM, and an IC50 of about 10 μM. The same inhibitory profiles of tetrapeptide inhibitors on filamin cleavage were also seen in the case of Jurkat cells (data not shown). These results suggest that a caspase-3–like activity, but not a caspase-1–like activity, is required for filamin cleavage (*40*).

It is known that caspase-3 plays an important role in the execution of apoptosis, and in this system, caspase-3 was proteolytically cleaved and activated as described above. Therefore we tested whether caspase-3 could produce filamin fragments of the same sizes as seen in U937 and Jurkat cells during apoptosis. We prepared a recombinant active form of caspase-3 directly using a *Pichia pastoris* overexpression system. The cleavage of filamin by recombinant caspase-3 was assessed by Western blot analysis using the anti-filamin antibodies MAb PM6/317 (Fig. 5, A and C) and MAb Tl10 (Fig. 5, B and D). Incubation of non-

treated U937 and Jurkat cell lysates with recombinant caspase-3 resulted in cleavage to produce fragments identical in size to those observed during the apoptosis of U937 (Fig. 5, A and B) and Jurkat cells (Fig. 5, C and D). To confirm further the specific cleavage of filamin by caspase-3, we examined peptide inhibitors for their ability to inhibit this cleavage. The tetrapeptide aldehyde Ac-DEVD-cho completely inhibited cleavage at concentrations lower than 10 μM (Fig. 5, A–D). In apoptotic U937 cells, the generation of the 170 kDa N-terminal fragment preceded that of the 120 kDa fragment, and the 150 kDa N-terminal fragments appeared last (Fig. 5A). In Jurkat cells, the 170 and 150 kDa N-terminal fragments were detected simultaneously, with the 120 kDa N-terminal fragment following soon after (Fig. 5C). Therefore, the cleavage pattern of the C-terminal fragments were also the same as observed in apoptotic cells *in vivo* (Fig. 5, B and D). The 110 kDa C-terminal fragment formed in Jurkat cells *in vitro* is thought to be generated because the caspase-3 activity is stronger than that observed in apoptotic cells (Fig. 5D). These results demonstrate that caspase-3 is capable of specifically cleaving filamin to produce fragments identical to those seen in apoptosis-induced cells *in vivo*.

We then examined whether filamin from human platelets can be directly cleaved by caspase-3 *in vivo*. Platelet filamin was digested with caspase-3. Immunoblot analysis using anti-filamin antibody showed that the partially purified 280 kDa filamin was cleaved to 170, 150, and 120 kDa N-terminal fragments with a similar pattern to that observed in etoposide-induced apoptotic U937 and Jurkat

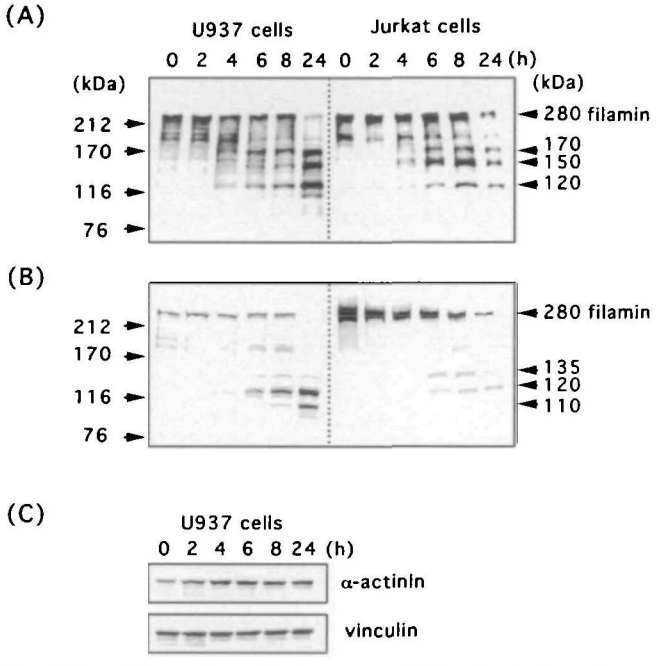

Fig. 2. **Specific and rapid cleavage of filamin during etoposide-induced apoptosis.** U937 and Jurkat cells were treated with 10 μg/ml etoposide for 0, 2, 4, 6, 8, or 24 h. Cell lysates were prepared and analyzed by Western blotting using anti-filamin antibodies: MAb PM6/317 against the 190 kDa N-terminal fragments catalyzed by calpain (A); or MAbTl10 against the 90 kDa C-terminal fragments catalyzed by calpain (B), and anti-α-actinin, or vinculin (C), as described in "MATERIALS AND METHODS." Intact filamin migrates at a molecular mass of 280 kDa. The arrowheads on the right indicate the 170, 150, and 120 kDa N-terminal cleavage fragments, and, 135, 120, and 110 kDa C-terminal cleavage fragments. Molecular mass standards are indicated by the arrowheads on the left.

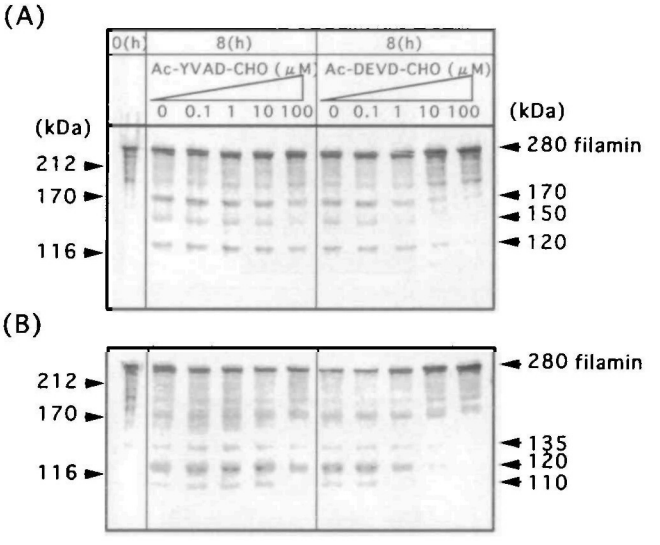

Fig. 4. **The cleavage of filamin during apoptosis is prevented by pretreatment with Ac-DEVD-cho, a caspase-3–like protease inhibitor.** U937 cells were preincubated for 1 h with the indicated concentrations of Ac-DEVD-cho, a caspase-3–like protease inhibitor, or Ac-YVAD-cho, a caspase-1–like protease inhibitor, and then stimulated with 10 μg/ml of etoposide for an additional 8 h at 37°C. For the determination of filamin cleavage, cell lysates prepared from etoposide-treated cells were analyzed by Western blotting with anti-filamin antibodies: MAb PM6/317 (A) or MAbTl10 (B). The arrowheads at the right indicate the cleavage products of native filamin.

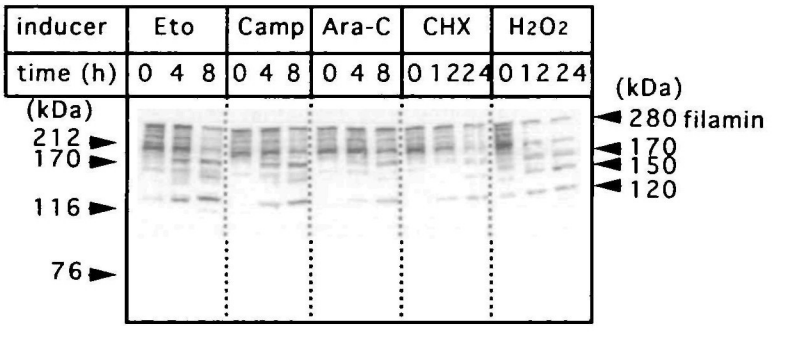

Fig. 3. **Cleavage of filamin in U937 cells during chemotherapeutic agent–induced apoptosis.** U937 cells were treated with 10 μg/ml etoposide (Eto), 10 μg/ml camptothecin (Camp), or 10 μg/ml Ara-C for 0, 4, or 8 h, or, 10 μg/ml cycloheximide (CHX) or 10 μM H_2O_2 for 0, 12, or 24 h.

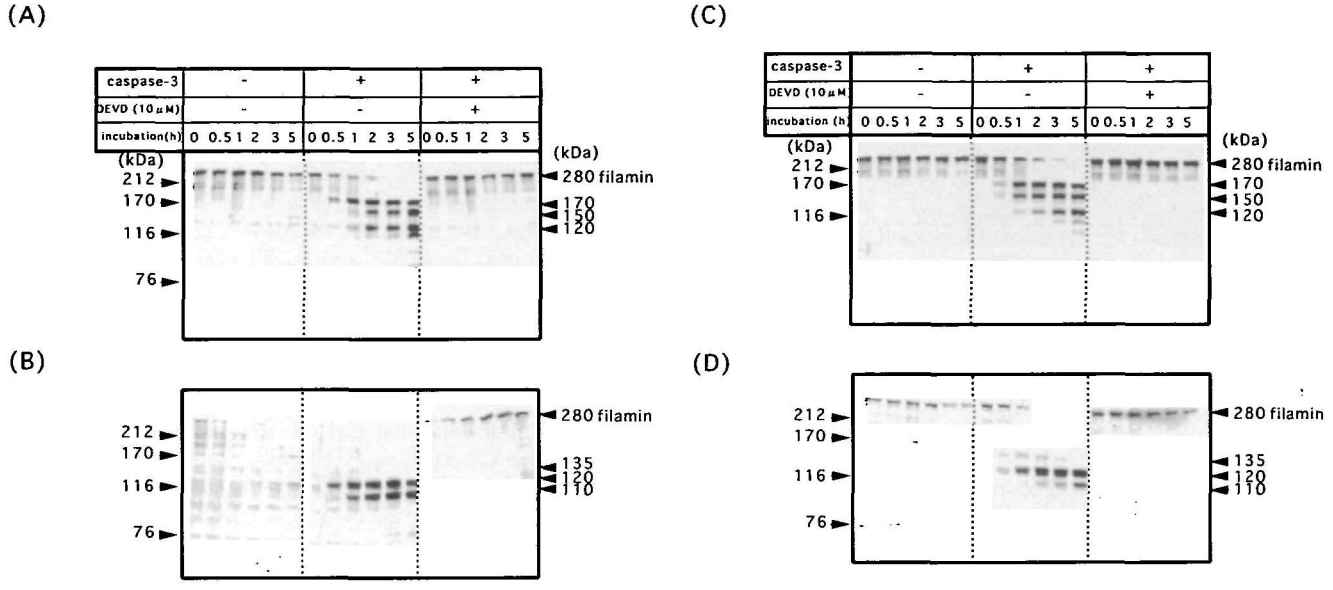

Fig. 5. **Filamin is cleaved by recombinant caspase-3 *in vitro* and yields the same size fragments as seen in apoptotic U937 and Jurkat cells *in vivo*.** Non-apoptotic extracts prepared from U937 (A, B) and Jurkat cells (C, D) were incubated with or without recombinant caspase-3 and 10 μM of a tetrapeptide inhibitor, Ac-DEVD-cho, at 37°C for the indicated times and analysed by immunoblotting with anti-filamin antibodies: MAb PM6/317 (A, C) or MAb Tl10 (B, D). With recombinant caspase-3, both *in vitro* cleavage systems yield the same length cleavage fragments as seen in apoptotic U937 and Jurkat cells *in vivo*, as indicated by the arrowheads at the right.

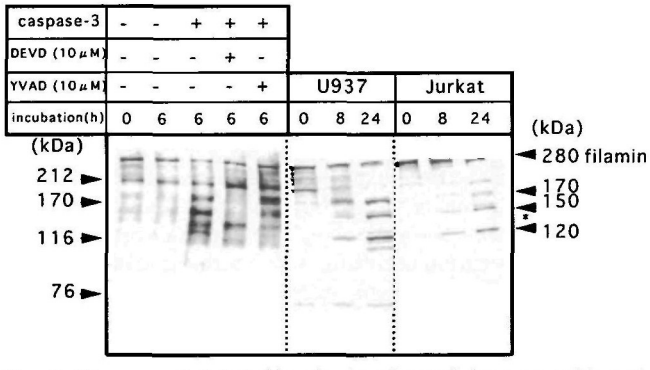

Fig. 6. **Human platelet filamin is cleaved by recombinant caspase-3.** Platelet filamin was incubated with or without recombinant caspase-3 and 10 μM of the tetrapeptide inhibitor Ac-DEVD-cho or Ac-YVAD-cho at 37°C for the indicated times, and analysed by immunoblotting with anti-filamin antibody, MAb PM6/317. The arrowheads indicate the caspase-3 catalyzed products, and nonspecific products (*) generated in the *in vitro* assay that are not dependent on capsase-3. Using recombinant caspase-3, both *in vitro* cleavage systems yield the same length cleavage fragments as seen in apoptotic U937 and Jurkat cells *in vivo*, as indicated by arrowheads.

cells *in vivo* (Fig. 6). The above results clearly demonstrate that caspase-3 directly catalyzes the limited proteolysis of filamin during apoptosis.

DISCUSSION

We have demonstrated that filamin, an abundant cytoskeletal protein, is specifically cleaved during the process of chemotherapeutic agent–induced apoptosis, and that this cleavage is catalyzed by caspase-3, generating 170, 150, and 120 kDa N-terminal fragments and 135, 120, and 110 kDa C-terminal fragments. We found that this cleavage proceeds in a similar manner among different cell lines and various stimuli induced by pharmacological reagents. Filamin is known to form homodimers in non-muscle cells and to regulate the three-dimensional organization of actin filaments in the submembranous cortex. The N-terminal region of filamin contains an actin binding domain, followed by a semiflexible rod-like domain consisting of 24 tandem repeats, each approximately 96 amino acids in length. Each domain is predicted to have six to eight short β-sheets, and these repeats interact intramolecularly to form a rigid rod-like structure (*37*) (Fig. 7).

It has been reported that filamin is cleaved by calpain *in vivo*. For example, during platelet activation, calpain is autocatalytically processed and activated by an elevation in the cellular calcium concentration, and filamin is cleaved and loses its ability to bundle F-actin (*41*). On the other hand, calcium elevation also leads to calpain activation in the process of apoptosis (*42*); however, the filamin cleavage pattern is obviously different from the calpain–mediated degradation described previously (*37–39*). Therefore, this event appears to be specific for apoptosis. In fact, we showed directly that this cleavage is catalyzed by caspase-3 using an *in vitro* cell-free assay system. Also the models for the limited cleaved products *in vitro* were the same as those observed in apoptotic human cell lines *in vivo*. Considering the elevation in the caspase-3–like activity in apoptotic cells, these findings suggest that caspase-3, but not calpain, may be responsible for filamin proteolysis.

In this study, we focused on filamin proteolysis in floating cell lines and showed their common cleavage properties during apoptosis. In contrast, it remains unknown whether these phenomena are applicable to apoptosis in adherent

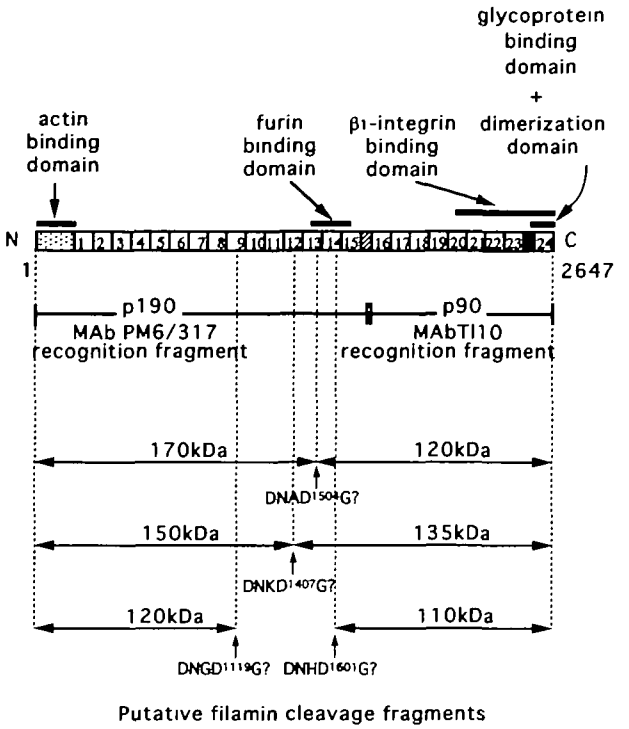

Fig. 7. **Schematic representation of the structure of filamin with the predicted cleavage sites and functional domains.** The recognition sites of the anti-filamin antibodies are indicated. The putative fragments generated by caspase-3–catalyzed cleavage during etoposide-induced apoptosis are indicated.

cells. Kothakota *et al.* showed that gelsolin but not filamin undergoes proteolysis during apoptosis in murine fibroblast L929 cells stably expressing a chimeric receptor composed of the extracellular and transmembrane domains of murine CD4 and the cytoplasmic domain of Fas (*43*). To address whether these phenomena commonly occur in adherent cells, we chose C2C12 cells, derived from a mouse myogenic cell line. Like other adherent cells, C2C12 showed no morphological changes or DNA fragmentation when exposed to apoptotic stimuli. In contrast, C2C12 cells transfected with MtPK (myotonin protein kinase) containing CTG triplet repeats at the 3′ untranslated region underwent apoptosis by etoposide and methylmercury (*44*). Although it is unknown how these transformants gain susceptibility to apoptotic stimuli, we monitored the occurrence of DNA fragmentation. In contrast to floating cells, the elevation in the caspase-3–like activity in C2C12 cells was less, and no limited proteolysis of filamin was observed (data not shown).

These results suggest that caspase-3 or a caspase-3–like activity correlates with filamin proteolysis in the process of apoptosis. However, it is impossible to exclude the possibilities that another homologue of filamin (resistant to caspase digestion) exists in adherent cells, or that various phosphorylated states of filamin in different cell lines alter the susceptibility to caspase digestion. For example, the phosphorylation of filamin by PKA is resistant to calpain proteolytic cleavage (*45, 46*).

An increasing number of caspase-3 substrates cleaved at the DXXD consensus sequence have been identified. According to the primary sequence, filamin has 22 putative caspase-3 cleavage sites (*37, 47*). Specifically, there are numerous DXXD consensus sequences between the 5th and 6th β-sheets in each segment. But the full-length 280 kDa filamin is specially cleaved to 170, 150, and 120 kDa N-terminal fragments, and 135, 120, and 110 kDa C-terminal fragments. The time course for the appearance of these fragments suggests that there is a hierarchy in the proteolytic cascade, perhaps in part because not all potential cleavage sites are cleaved by caspase-3. For example, a 170 kDa C-terminal fragment and a 120 kDa N-terminal fragment appear at an early stage (Fig. 2, A and B). To estimate the cleavage sites, we diagrammed the human filamin structure and predicted cleaved fragments (Fig. 7). The actin binding domain is near the N-teminus; the furin binding domain is near the middle; the β1-integrin and glycoprotein binding domains, and the self-dimerization domain are near the C-terminus of filamin. Calpain is known to cleave at the hinge region between the 15th and 16th segments to generate a 190 kDa N-terminal fragment and a 90 kDa C-terminal fragment. Considering the potential sites for filamin proteolysis, it is reasonable to predict that the DNAD^{1504}G sequence in the 13th segment would be preferentially cleaved. Cleavage at this site is assumed to generate a 170 kDa N-terminal fragment and a 120 kDa C-terminal fragment. We assumed that cleavage of the C-terminal fragment at the DNHD^{1601}G in the 14th segment generates the 110 kDa fragment, because no DXXD sequence is found in the C-terminus of the 120 kDa fragment. DNKD^{1407}G in the 12th segment is assumed to be subject to further proteolysis resulting in the generation of a 150 kDa C-terminal fragment and a 135 kDa N-terminal fragment. And a 120 kDa N-terminal fragment is assumed to be formed by cleavage at DNGD^{1119}G in the 9th segment, because an appropriate DXXD motif is not found in the N-terminus of filamin.

Although the physiological significance of the limited proteolysis of cytoskeletal proteins has not been clarified, it is postulated that these proteolytic events disrupt the cytoskeletal organization resulting in the formation of apoptotic bodies. Therefore, it is meaningful that large molecules such as spectrin and filamin are cleaved and produce fragments. Also, gelsolin is cleaved and activated, and then cleaves actin filaments. However, α-actinin, which bundles actin filaments, and vinculin, which binds to actin filaments mediated by α-actinin, are not cleaved during apoptosis.

Recently, Browne *et al.* reported that filamin is cleaved by z-Val-Ala-Asp-fluoromethylketone–sensitive caspases during anti-Fas antibody–induced apoptosis of Jurkat cells, and that granzyme B cleaves filamin in a caspase-independent manner (*32*). Moreover, filamin-deficient human melanoma cells were protected from granzyme B–mediated death. These results suggest that toxic cleavage products may be generated from filamin by granzyme B, but there is no evidence that filamin cleavage products are toxic. Therefore it will be important to determine cleavage sites by caspase-3 and granzyme B, and then compare the physiological meaning of filamin proteolysis.

Filamin is commonly expressed in various cell lines and many tissues. It is postulated that its function is not only to maintain the cell structure, but also to bind many signaling molecules (*48–51*). Moreover, Fox *et al.* reported that mutations in filamin lead to the X-linked dominant human dis-

ease periventricular heterotopia, which prevents the migration of cerebral cortical neurons, and lymphoid depletion of the thymus (*52*), and is considered relevant to cell death. These findings indicate filamin is a very important molecule as the target of multiple cytoplasmic signaling proteins that control intracellular communication and differentiation. Hence, the loss of filamin function causes severe defects in embryonic development, especially in males (*52*). Next, we will try to elucidate the physiological function of filamin proteolysis in immune systems.

REFERENCES

1. Wyllie, A.H., Kerr, J.F., and Currie, A.R. (1980) Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68**, 251–306
2. Smith, C.A., Williams, G.T., Kingston, R., Jenkinson, E.J., and Owen, J.J. (1989) Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature* **337**, 181–184
3. Arends, M.J. and Wyllie, A.H. (1991) Apoptosis: mechanisms and roles in pathology. *Int. Rev. Exp. Pathol.* **32**, 223–254
4. Raff, M.C. (1992) Social controls on cell survival and cell death. *Nature* **356**, 397–400
5. Cohen, J.J., Duke, R.C., Fadok, V.A., and Sellins, K.S. (1992) Apoptosis and programmed cell death in immunity. *Annu. Rev. Immunol.* **10**, 267–293
6. Takahashi, A. and Earnshaw, W.C. (1996) ICE-related proteases in apoptosis. *Curr. Opin. Genet. Dev.* **6**, 50–55
7. Ellis, H.M. and Horvitz, H.R. (1986) Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* **44**, 817–829
8. Hengartner, M.O. and Horvitz, H.R. (1994) *C. elegans* cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell* **76**, 665–676
9. Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M., and Horvitz, H.R. (1993) The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* **75**, 641–652
10. Wang, L., Miura, M., Bergeron, L., Zhu, H., and Yuan, J. (1994) Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell* **78**, 739–750
11. Fernandes-Alnemri, T., Litwack, G., and Alnemri, E.S. (1994) CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. *J. Biol. Chem.* **269**, 30761–30764
12. Tewari, M., Quan, L.T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D.R., Poirier, G.G., Salvesen, G.S., and Dixit, V.M. (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitate protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81**, 801–809
13. Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., Munday, N.A., Raju, S.M., Smulson, M.E., Yamin, T-T., Yu, V.L., and Miller, D.K. (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376**, 37–43
14. Faucheu, C., Diu, A., Chan, A.W., Blanchet, A.M., Miossec, C., Herve, F., Collard-Dutilleul, V., Gu, Y., Aldape, R.A., and Lipke, J.A. (1995) A novel human protease similar to the interleukin-1 beta converting enzyme induces apoptosis in transfected cells. *EMBO J.* **14**, 1914–1922
15. Munday, N.A., Vaillancourt, J.P., Ali, A., Casano, F.J., Miller, D.K., Molineaux, S.M., Yamin, T.T., Yu, V.L., and Nicholson, D.W. (1995) Molecular cloning and pro-apoptotic activity of ICErelII and ICErelIII, members of the ICE/CED-3 family of cysteine proteases. *J. Biol. Chem.* **270**, 15870–15876
16. Kamens, J., Paskind, M., Hugunin, M., Talanian, R.V., Allen, H., Banach, D., Bump, N., Hackett, M., Johnston, C.G., Li, P., Mankovich, J.A., Terranova, M., and Ghayur, T. (1995) Identification and characterization of ICH-2, a novel member of the interleukin-1 β-converting enzyme family of cysteine proteases. *J. Biol. Chem.* **270**, 15250–15256
17. Fernandes-Alnemri, T., Takahashi, A., Armstrong, R., Krebs, J., Fritz, L., Tomaselli, K.J., Wang, L., Yu, Z., Croce, C.M., and Salveson, G. (1995) Mch3, a novel human apoptotic cysteine protease highly related to CPP32. *Cancer Res.* **55**, 6045–6052
18. Fernandes-Alnemri, T., Litwack, G., and Alnemri, E.S. (1995) Mch2, a new member of the apoptotic Ced-3/Ice cysteine protease gene family. *Cancer Res.* **55**, 2737–2742
19. Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., and Yuan, J. (1996) Human ICE/CED-3 protease nomenclature. *Cell* **87**, 171
20. Duan, H., Chinnaiyan, A.M., Hudson, P.L., Wing, J.P., He, W.W., and Dixit, V.M. (1996) ICE-LAP3, a novel mammalian homologue of the *Caenorhabditis elegans* cell death protein Ced-3 is activated during Fas- and tumor necrosis factor-induced apoptosis. *J. Biol. Chem.* **271**, 1621–1625
21. Duan, H., Orth, K., Chinnaiyan, A.M., Poirier, G.G., Froelich, C.J., He, W.W., and Dixit, V.M. (1996) ICE-LAP6, a novel member of the ICE/Ced-3 gene family, is activated by the cytotoxic T cell protease granzyme B. *J. Biol. Chem.* **271**, 16720–16724
22. Lippke, J.A., Gu, Y., Sarnecki, C., Caron, P.R., and Su, M.S. (1996) Identification and characterization of CPP32/Mch2 homolog 1, a novel cysteine protease similar to CPP32. *J. Biol. Chem.* **271**, 1825–1828
23. Wang, S., Miura, M., Jung, Y.K., Zhu, H., Gagliardini, V., Shi, L., Greenberg, A.H., and Yuan, J. (1996) Identification and characterization of Ich-3, a member of the interleukin-1beta converting enzyme (ICE)/Ced-3 family and an upstream regulator of ICE. *J. Biol. Chem.* **271**, 20580–20587
24. Duan, H. and Dixit, V.M. (1997) RAIDD is a new 'death' adaptor molecule. *Nature* **385**, 86–89
25. Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V.M., and Salvesen, G.S. (1997) Target protease specificity of the viral serpin CrmA. Analysis of five caspases. *J. Biol. Chem.* **272**, 7797–7800
26. Humke, E.W., Ni, J., and Dixit, V.M. (1998) ERICE, a novel FLICE-activatable caspase. *J. Biol. Chem.* **273**, 15702–15707
27. Hu, S., Snipas, S.J., Vincenz, C., Salvesen, G., and Dixit, V.M. (1998) Caspase-14 is a novel developmentally regulated protease. *J. Biol. Chem.* **273**, 29648–29653
28. Oberhammer, F.A., Hochegger, K., Froschl, G., Tiefenbacher, R., and Pavelka, M. (1994) Chromatin condensation during apoptosis is accompanied by degradation of lamin A+B, without enhanced activation of cdc2 kinase. *J. Cell Biol.* **126**, 827–837
29. 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
30. Kouchi, Z., Saido, T.C., Ohyama, H., Maruta, H., Suzuki, K., and Tanuma, S. (1997) The restrictive proteolysis of alpha-fodrin to a 120 kDa fragment is not catalyzed by calpains during thymic apoptosis. *Apoptosis* **2**, 84–90
31. 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
32. Browne, K.A., Johnstone, R.W., Jans, D.A., and Trapani, J.A. (2000) Filamin (280-kDa actin-binding protein) is a caspase substrate and is also cleaved directly by the cytotoxic T lymphocyte protease granzyme B during apoptosis. *J. Biol. Chem.* **275**, 39262–39266
33. Koriyama, H., Kouchi, Z., Umeda, T., Saido, T.C., Momoi, T., Ishiura, S., and Suzuki, K. (1999) Proteolytic activation of protein kinase C delta and epsilon by caspase-3 in U937 cells during chemotherapeutic agent-induced apoptosis. *Cell. Signal.* **11**, 831–838
34. Feramisco, J.R. and Burridge, K. (1980) A rapid purification of alpha-actinin, filamin, and a 130,000-dalton protein from smooth muscle. *J. Biol. Chem.* **255**, 1194–1199

35. Cryns, V.L., Bergeron, L., Zhu, H., Li, H., and Yuan, J. (1996) Specific cleavage of alpha-fodrin during Fas- and tumor necrosis factor-induced apoptosis is mediated by an interleukin-1beta-converting enzyme/Ced-3 protease distinct from the poly-(ADP-ribose) polymerase protease. *J. Biol. Chem.* **271**, 31277–31282
36. Wang, K.K., Posmantur, R., Nath, R., McGinnis, K., Whitton, M., Talanian, R.V., Glantz, S.B., and Morrow, J.S. (1998) Simultaneous degradation of alphaII- and betaII-spectrin by caspase 3 (CPP32) in apoptotic cells. *J. Biol. Chem.* **273**, 22490–22497
37. Gorlin, J.B., Yamin, R., Egan, S., Stewart, M., Stossel, T.P., Kwiatkowski, D.J., and Hartwig, J.H. (1990) Human endothelial actin-binding protein (ABP-280, nonmuscle filamin): a molecular leaf spring. *J. Cell Biol.* **111**, 1089–1105
38. Kwak, K.B., Chung, S.S., Kim, O.M., Kang, M.S., Ha, D.B., and Chung, C.H. (1993) Increase in the level of m-calpain correlates with the elevated cleavage of filamin during myogenic differentiation of embryonic muscle cells. *Biochim. Biophys. Acta* **1175**, 243–249
39. Basse, F., Gaffet, P., and Bienvenue, A. (1994) Correlation between inhibition of cytoskeleton proteolysis and anti-vesiculation effect of calpeptin during A23187-induced activation of human platelets: are vesicles shed by filopod fragmentation? *Biochim. Biophys. Acta* **1190**, 217–224
40. Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T., and Nicholson, D.W. (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J. Biol. Chem.* **272**, 17907–17911
41. Onji, T., Takagi, M., and Shibata, N. (1987) Calpain abolishes the effect of filamin on the actomyosin system in platelets. *Biochim. Biophys. Acta* **912**, 283–286
42. Squier, M.K., Miller, A.C., Malkinson, A.M., and Cohen, J.J. (1994) Calpain activation in apoptosis. *J. Cell Physiol.* **159**, 229–237
43. Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., McGarry, T.J., Kirschner, M.W., Koths, K., Kwiatkowski, D.J., and Williams, L.T. (1997) Calpain activation in apoptosis. *Science* **278**, 294–298
44. Usuki, F., Takahashi, N., Sasagawa, N., and Ishiura, S. (2000) Differential signaling pathways following oxidative stress in mutant myotonin protein kinase cDNA-transfected C2C12 cell lines. *Biochem. Biophys. Res. Commun.* **267**, 739–743
45. Chen, M. and Stracher, A. (1989) In situ phosphorylation of platelet actin-binding protein by cAMP-dependent protein kinase stabilizes it against proteolysis by calpain. *J. Biol. Chem.* **264**, 14282–14289
46. Jay, D., Garcia, E.J., Lara, J.E., Medina, M.A., and de la Luz Ibarra, M. (2000) Determination of a cAMP-dependent protein kinase phosphorylation site in the C-terminal region of human endothelial actin-binding protein. *Arch. Biochem. Biophys.* **377**, 80–84
47. Cohen, G.M. (1997) Caspases: the executioners of apoptosis. *Biochem. J.* **326**, 1–16
48. Sharma, C.P., Ezzell, R.M., and Arnaout, M.A. (1995) Direct interaction of filamin (ABP-280) with the beta 2-integrin subunit CD18. *J. Immunol.* **154**, 3461–3470
49. Zhang, W., Han, S.W., McKeel, D.W., Goate, A., and Wu, J.Y. (1996) Interaction of presenilins with the filamin family of actin-binding proteins. *J. Neurosci.* **18**, 914–922
50. Edwards, D.N., Towb, P., and Wasserman, S.A. (1997) An activity-dependent network of interactions links the Rel protein Dorsal with its cytoplasmic regulators. *Development* **124**, 3855–3864
51. Marti, A., Luo, Z., Cunningham, C., Ohta, Y., Hartwig, J., Stossel, T.P., Kyriakis, J.M., and Avruch, J. (1997) Actin-binding protein-280 binds the stress-activated protein kinase (SAPK) activator SEK-1 and is required for tumor necrosis factor-alpha activation of SAPK in melanoma cells. *J. Biol. Chem.* **272**, 2620–2628
52. Fox, J.W., Lamperti, E.D., Eksioglu, Y.Z., Hong, S.E., Feng, Y., Graham, D.A., Scheffer, I.E., Dobyns, W.B., Hirsch, B.A., Radtke, R.A., Berkovic, S.F., Huttenlocher, P.R., and Walsh, C.A. (1998) Mutations in filamin 1 prevent migration of cerebral cortical neurons in human periventricular heterotopia. *Neuron* **21**, 1315–1325