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Specific Cleavage of α-Fodrin during Fas- and Tumor Necrosis Factor-induced Apoptosis Is Mediated by an Interleukin-1β-converting Enzyme/Ced-3 Protease Distinct from the Poly(ADP-ribose) Polymerase Protease

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Interleukin-1β-converting enzyme (ICE)/Ced-3 proteases play a critical role in apoptosis. One well characterized substrate of these proteases is the DNA repair enzyme poly(ADP-ribose) polymerase. We report here that α-fodrin, an abundant membrane-associated cytoskeletal protein, is cleaved rapidly and specifically during Fas- and tumor necrosis factor-induced apoptosis; this cleavage is mediated by an ICE/Ced-3 protease distinct from the poly(ADP-ribose) polymerase protease. Studies in cells treated with these apoptotic stimuli reveal that both fodrin and poly(ADP-ribose) polymerase proteolysis are inhibited by acetyl-Tyr-Val-Ala-Asp chloromethyl ketone and CrmA, specific inhibitors of ICE/Ced-3 proteases. However, fodrin proteolysis can be distinguished from poly(ADP-ribose) polymerase proteolysis by its relative insensitivity to acetyl-Asp-Glu-Val-Ala-Asp chloromethyl ketone and CrmA, specific inhibitors of ICE/Ced-3 proteases. Moreover, purified fodrin is cleaved in vitro by CPP32 (but not by ICE) into fragments of the same size observed in vivo during apoptosis. These findings suggest that fodrin proteolysis in vivo may reflect the activity of multiple ICE/Ced-3 proteases whose partial sensitivity to DEVD-CHO reflects a limited contribution from CPP32, or an ICE/Ced-3 protease less sensitive than CPP32 to DEVD-CHO inhibition.

Proteases of the interleukin-1β-converting enzyme (ICE)†‡

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†The abbreviations used are: ICE, interleukin-1β-converting enzyme; interleukin-1β; IL-1β; TNF, tumor necrosis factor; PARP, poly(ADP-ribose) polymerase; FCS, fetal calf serum; YVAD-CMK, acetyl-Tyr-Val-Ala-Asp chloromethyl ketone; DEVD-CHO, acetyl-Asp-Glu-Val-Ala-Asp chloromethyl ketone; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; CHX, cycloheximide; mAb, monoclonal antibody.

Ced-3 family are homologs of the Caenorhabditis elegans Ced-3 gene product, a protein whose function is required for programmed cell death during nematode development. ICE was the first mammalian homolog of Ced-3 to be identified; this novel cysteine protease cleaves pro-interleukin-1β (pro-IL-1β) into the mature cytokine (1, 2). Subsequently, several additional members of this rapidly growing family have been identified: Nedd2/Ich-1 (3–5), CPP32/Yama/Apopain (6–8), ICH-2/TX/ICE rel-II (9–11), ICE rel-III (11), Mch2 (12), Mch3/ICE-LAP3/CM1-I (13–15), and MACH/FLICE (16, 17). All members of the ICE/Ced-3 family are synthesized as inactive proenzymes that are activated by proteolytic cleavage, and they specifically cleave substrates (including themselves) C-terminally as aspartic acid residue at the P1 site (reviewed in Ref. 18).

Several lines of evidence suggest that proteases of the ICE/Ced-3 family play a critical role in the execution of apoptosis. Ectopic expression of cDNAs encoding these proteases induces programmed cell death (4–6, 9–12, 14, 15, 19). CrmA, a serpin encoded by cowpox virus that inhibits ICE (20), delays or prevents apoptosis induced by nerve growth factor withdrawal (21), tumor necrosis factor-α (TNF-α) (22–24), anti-Fas antibody (22, 23, 25), or disruption of the extracellular matrix (26). Moreover, specific peptide inhibitors of ICE/Ced-3 proteases suppress apoptosis induced by anti-Fas antibody (23, 25, 27, 28), neurotrophic factor deprivation (29), etoposide and glucocorticoids (30), and motor neuron cell death in vivo during development (29).

Since ICE/Ced-3-related proteases are a family of proteases, the identification and characterization of their physiological substrates are essential to understand their mechanism of action. Several substrates of these proteases have been identified. One such substrate is the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) (31, 32). PARP is cleaved rapidly and specifically during apoptosis (22, 31, 32) at a site (DEVD 216-G 217) between its DNA binding and catalytic domains (33). Studies in cell-free systems revealed that the PARP protease is an ICE/Ced-3 protease distinct from ICE, inhibited by micromolar concentrations of YVAD-CMK (33), a peptide inhibitor modeled after the pro-IL-1β cleavage sequence (1), or by nanomolar concentrations of DEVD-CHO, a peptide inhibitor containing the PARP cleavage sequence (8). Importantly, these inhibitors also prevent apoptosis (8, 23, 28, 33). Recent work has implicated CPP32/Yama/Apopain (6–8) as a major component of the PARP protease, although other ICE/Ced-3 proteases may also contribute to PARP proteolysis during apoptosis (12, 13, 15, 34). Other substrates of ICE/Ced-3 proteases cleaved during programmed cell death include the sterol regulatory element-binding proteins (35, 36), the nuclear laminas...
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(37), the 70-kDa protein component of the U1 small nuclear ribonucleoprotein (38, 39), the retinoblastoma tumor suppressor gene product (40), protein kinase C (41), DNA-dependent protein kinase (42), and the GDP dissociation inhibitor D4-GDI (43). Although each of these proteins has been shown to be cleaved by ICE/Ced-3 proteases during apoptosis, the functional significance of these cleavage events remains largely unknown.

Proteolysis of fodrin (non-erythroid spectrin) has been reported during apoptosis induced by a variety of stimuli (44). Fodrin is an abundant and highly conserved cytoskeletal protein consisting of α and β subunits. Apoptotic cleavage of the 240-kDa α-fodrin subunit into 150- and 120-kDa fragments has been attributed to the proteolytic action of calpain I, based on the ability of a peptide antibody generated against the calpain I-fodrin cleavage sequence to recognize these fragments in apoptotic cells (44). In this report, we demonstrate that specific α-fodrin cleavage occurs rapidly during Fas- and TNF-induced cell death and is mediated by an ICE/Ced-3 protease (or proteases) that can be distinguished from the PARP protease by its insensitivity to DEVD-CHO. In addition, we show that the fodrin protease is entirely resistant to a panel of calpain inhibitors. The observation that fodrin proteolysis can occur in cells protected from undergoing apoptosis by DEVDD-CHO indicates that cleavage of this protein can be uncoupled from apoptotic cell death.

EXPERIMENTAL PROCEDURES

Reagent—Calpain inhibitor I/N-acetyl-leucyl-leucyl-norleucinal (LlNaL), calpain inhibitor II/N-acetyl-leucyl-leucyl-methioninal (LlLM), tosyl-lysine chloromethyl ketone (TLCK), tosyl-phenylalanine chloromethyl ketone (TPCK), antipain dihydrochloride, E-64, and leupeptin were purchased from Boehringer Mannheim. Acetyl-Tyr-Val-Ala-Asp chloromethyl ketone (YVAD-CMK) and acetyl-Asp-Glu-Val-Asp aldehyde (DEVD-CHO) were obtained from Bachem Biosciences, Inc. Phenylmethylsulfonyl fluoride (PMSF), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and all other molecular biology grade reagents were purchased from Sigma. Stock solutions were prepared according to the manufacturer’s instructions.

Cell Lines—Jurkat cells were grown in RPMI 1640 medium (Life Technologies, Inc.) with 10% fetal calf serum (FCS). HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) and 10% FCS at densities of 5 × 10⁴ cells/well, respectively, in 96-well plates. For time-course analyses, Jurkat and HeLa cell lines were described previously (24). Jurkat and HeLa cell lines were transfected with pET-15b expression vector (Novagen). These plasmids were transformed into Escherichia coli strain BL21(DE3), and exponentially growing bacteria were inoculated with 0.2 mM isopropyl-1-thio-β-galactopyranoside for 2 h, harvested, and lysed by sonication in a buffer containing 0.5% Nonidet P-40, 20 mM HEPES (pH 7.4), and 100 mM NaCl. The lysates were cleared by centrifugation and stored in aliquots at −70 °C. The total protein content of these bacterial lysates was determined by Bio-Rad protein assay according to the manufacturer’s instructions.

In Vitro Cleavage Reactions—pBSK-pro-IL-1β and pBSK-PARP were transcribed and translated in vitro using the TNT coupled transcription/translation kit (Promega) in the presence of [35S]methionine. Purified bovine brain fodrin was a generous gift from Drs. John Morrow and Susan Glantz. In vitro cleavage reactions were performed in a buffer containing 0.5% Nonidet P-40, 20 mM HEPES (pH 7.4), 100 mM NaCl, and 20 mM dithiothreitol for 1 h at 37 °C; reactions were terminated by the addition of protein lysis buffer and boiling the samples for 5 min. Products of the cleavage reactions were separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography following fixation of gels in 20% MeOH/10% acetic acid and vacuum drying (for 35S-labeled substrates) or (ii) Western blotting (for α-fodrin). 4 μl of ICE lysate (20.9 μg of total protein) and CPP32 lysate (25.7 μg of total protein) were then treated to cleave >95% of 35S-pro-IL-1β and 35S-PARP, respectively, when incubated at 37 °C for 1 h. Consequently, 100 ng of purified fodrin was incubated as above with 4 or 11 μl (excess) of ICE and CPP32 lysates, as well as 17 μl of control lysate (61.0 μg of total protein) derived from bacteria containing the empty pET-15b vector.

RESULTS

Fodrin, a ubiquitous membrane-associated cytoskeletal protein, has recently been implicated as a “death substrate” whose cleavage during apoptosis might play a role in the membrane blebbing characteristic of this process (44). Candidate death substrates should be cleaved rapidly during the execution of apoptosis. To begin evaluating the α-subunit of fodrin (α-fodrin) as a potential death substrate, we examined the time course of α-fodrin proteolysis during Fas- and TNF-induced cell death in Jurkat and HeLa cells, respectively (Fig. 1). Within 2 h after treating cells with either anti-Fas mAb or TNF-α/CHX, two specific α-fodrin cleavage fragments of approximately 150 and 120 kDa were readily discerned. At this 2-h time point, 94% of the Jurkat cells and approximately 100% of the HeLa cells were viable as determined by the MTT conversion assay, indicating that α-fodrin cleavage begins during the induction of

![Fig. 1. Specific and rapid cleavage of α-fodrin during Fas- and TNF-induced apoptosis.](http://www.jbc.org/)

% Viable 100 98 93 73 20

![Graph showing cell viability and protein levels](http://www.jbc.org/)
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Fig. 2. Different inhibitor profiles of fodrin and PARP proteolysis during apoptosis. A, different sensitivities of Fas-mediated α-fodrin and PARP cleavage to a panel of protease inhibitors. Jurkat cells were either untreated (UnRX) or preincubated for 2 h with H2O, 0.05% Me2SO (DMSO), 0.05% MeOH, DEVD-CMK (50 or 250 μM), YVAD-CMK (50 or 250 μM), LLM (25 or 200 μM), LLnL (25 or 200 μM), 200 μM E-64, 1 mM TLCK, or 200 μM TPCK, and then treated with 200 ng/ml anti-Fas mAb for 5 h. CrmA inhibits TNF-induced α-fodrin and PARP proteolysis. HeLa cells stably transfected with crmA were treated with 10 ng/ml TNF-α and 10 μg/ml CHX for 0, 4, 7, 5, and 23 h. Cell lysates were prepared and analyzed by Western blotting using an α-fodrin mAb (upper panel) and PARP mAb (lower panel) as detailed under “Experimental Procedures.” The corresponding viability for each lysate was determined by MTT analysis as described under “Experimental Procedures.”

apoptosis. Using these same cell lysates, PARP cleavage was also initiated at this same 2-h time point (data not shown). Within 23 h, all of the intact 240-kDa α-fodrin was cleaved into these two fragments and <20% of cells were still viable. At all time points examined, the extent of α-fodrin proteolysis correlated with the degree of cell death.

Having demonstrated that α-fodrin proteolysis is an early event in apoptosis, we next wanted to determine the sensitivity of Fas-mediated fodrin cleavage to a panel of protease inhibitors. To this end, we preincubated Jurkat cells for 2 h with a variety of protease inhibitors before treating them with anti-Fas mAb for 5 h. Jurkat cells were >80% viable when treated with each of these protease inhibitors (except TLCK, TPCK, and LLnL) for 7 h in the absence of anti-Fas mAb; only TLCK (1 mM), TPCK (200 μM), and LLnL (200 μM) induced significant cell death in the absence of anti-Fas mAb (data not shown). As shown in Fig. 2A (upper panel), α-fodrin proteolysis was completely prevented by 250 μM YVAD-CMK, a specific tetrapeptide inhibitor of ICE/Ced-3 family proteases that closely resembles an ICE cleavage sequence in pro-IL-1β, and by the serine protease inhibitors TLCK (1 mM) and TPCK (200 μM). At the concentrations used in this study (50–250 μM), YVAD-CMK inhibits at least several members of the ICE/Ced-3 family in vitro, including ICE, ICH-1, and CPP32 (data not shown). In contrast, DEVD-CHO, a tetrapeptide inhibitor that is identical to the CPP32 cleavage sequence in PARP, preferentially inhibits CPP32 and CPP32-like ICE/Ced-3 family proteases (8, 13, 15). Interestingly, 250 μM DEVD-CHO only partially inhibited α-fodrin cleavage, while lower concentrations had no effect. All other protease inhibitors examined did not prevent Fas-mediated α-fodrin cleavage, including the calpain inhibitors LLM (25 or 200 μM) and LLnL (25 or 200 μM), E-64 (200 μM), leupeptin (250 μM), lactacystin (25 μM, a proteasome inhibitor) (47), antipain (200 μM), and PMSF (1 mM) (data for the last four inhibitors not shown). These data indicate that the fodrin protease is either (i) an ICE/Ced-3 protease only partially sensitive to DEVD-CHO inhibition or (ii) a proteolytic enzyme downstream of such an ICE/Ced-3 protease that is resistant to many protease inhibitors, including calpain inhibitors.

To better characterize the fodrin protease, we used these same lysates to determine the sensitivity of Fas-mediated PARP proteolysis to an identical panel of protease inhibitors (Fig. 2A, lower panel), thereby allowing comparison of the fodrin and PARP proteases. Recent work suggests that a major PARP protease is the ICE/Ced-3 family member CPP32/Yama/Apopain (6–8, 33), although additional ICE/Ced-3 proteases may also contribute to PARP proteolysis during apoptosis (12, 13, 15, 34). Like fodrin proteolysis, PARP proteolysis is completely inhibited by YVAD-CMK, TLCK, and TPCK, and is resistant to calpain inhibitors and E-64. However, unlike fodrin proteolysis, PARP proteolysis is very sensitive to inhibition by DEVD-CHO; preincubation of Jurkat cells with 10 μM DEVD-CHO prevented >50% of PARP cleavage, while 50 μM DEVD-CHO completely inhibited PARP cleavage (see also Table I). As noted above, DEVD-CHO, at concentrations as high as 250 μM, only partially inhibited fodrin proteolysis. The dramatically different sensitivities of fodrin and PARP proteolysis to inhibition by DEVD-CHO suggest that these cleavage events are mediated by different ICE/Ced-3 family proteases.

Further evidence implicating an ICE/Ced-3 protease in fodrin proteolysis comes from the observation that CrmA, a cow-pox serpin that inhibits some ICE/Ced-3 proteases, prevented TNF-mediated α-fodrin proteolysis (Fig. 2B, upper panel). HeLa cells stably transfected with crmA (24) were treated with TNF-α and CHX for various lengths of time, and the lysates were examined for α-fodrin cleavage. Even after 23 h of exposure to TNF-α and CHX, very little proteolysis of α-fodrin was observed in HeLa/CrmA cells. In contrast, complete proteolysis of α-fodrin was noted in identically treated parental HeLa cells after a 23-h exposure to TNF-α and CHX (Fig. 1, right panel). Moreover, TNF-mediated PARP cleavage was completely inhibited by CrmA (Fig. 2B, lower panel); under these conditions, PARP proteolysis is complete by 23 h in parental HeLa cells (data not shown).

To examine whether the cleavage of α-fodrin and PARP are coupled to cell death, we determined the corresponding viability of cells used to make each of the lysates in Fig. 2 (indicated at the bottom). For Jurkat cells treated with anti-Fas mAb, the ICE/Ced-3 protease inhibitor YVAD-CMK (50 and 250 μM) prevented α-fodrin and PARP proteolysis and also protected the cells from dying (>90% viability compared with 43% viability in the control cells pretreated with 0.05% MeOH alone, the vehicle used to dissolve YVAD-CMK). In addition, YVAD-CMK inhibited Fas-induced internucleosomal DNA fragmentation (Fig. 3) and membrane blebbing (data not shown). Similarly, the serine protease inhibitors TLCK and TPCK prevented Fas-induced α-fodrin and PARP cleavage, as well as the DNA fragmentation (Fig. 3) and membrane blebbing (data not shown) characteristic of apoptosis. However, both TLCK and TPCK, under the conditions used in these experiments, induced cell death without causing DNA fragmentation (Fig. 3), thereby accounting for the diminished viability of Fas-treated Jurkat cells pretreated with these inhibitors (viabilities of 19 and 51%, respectively). Thus, these serine protease inhibitors are intrinsically cytotoxic; they induce necrosis in Jurkat
Jurkat cells were either untreated (UnRx) or preincubated for 2 h with various concentrations of DEVD-CHO and then treated for an additional 5 h with 200 ng/ml anti-Fas mAb. PARP cleavage percentages were determined on three independent lysates as detailed under “Experimental Procedures.” Viabilities were determined by MTT analysis on at least quadruplicate samples and were normalized with respect to the viability of untreated cells as described under “Experimental Procedures.” Data are means ± S.E.

|          | UnRx | DEVD-CHO (μM) |
|----------|------|---------------|
| PARP cleavage (%) | 2.1 ± 1.1 | 74.9 ± 12.0 |
| Viability (%)    | 100  | 42.1 ± 2.1   |

![Inhibitors of ICE/Ced-3 and serine proteases prevent Fas-induced internucleosomal DNA fragmentation.](http://www.jbc.org/)

![CPP32 cleaves α-fodrin in vitro and yields fragments of the same size seen in vivo in apoptotic Jurkat cells.](http://www.jbc.org/)

![Fig. 4. CPP32 cleaves α-fodrin in vitro and yields fragments of the same size seen in vivo in apoptotic Jurkat cells. A, comparison of α-fodrin cleavage in apoptotic cells and in vitro by bacterially expressed ICE and CPP32. Cell lysates from untreated Jurkat cells (lane 1) and Jurkat cells treated with 200 ng/ml anti-Fas mAb for 5 h (lane 2) were used to indicate the size of intact α-fodrin (lane 1) and the apoptotic cleavage fragments (lane 2) in vivo. For the in vitro cleavage reactions, 100 ng of purified bovine fodrin was incubated for 1 h at 37 °C with H2O (lane 3), 17 μl of lysate (61.0 μg of total protein) of pET-15b vector-transformed bacteria (lane 4), 4 μl (20.9 μg of total protein) of ICE lysate (lane 5), 17 μl (88.7 μg of total protein) of ICE lysate (lane 6), 4 μl (25.7 μg of total protein) of CPP32 lysate (lane 7), or 17 μl (109.3 μg of total protein) CPP32 lysate. The cell lysates and the products of the in vitro cleavage reactions were analyzed by Western blotting using an α-fodrin mAb as described under “Experimental Procedures.” B, bacterially expressed ICE (left panel) and CPP32 (right panel) are active. In vitro translated 35S-pro-IL-1β and 35S-PARP were incubated with 17 μl of lysate of pET-15b vector-transformed bacteria and 4 μl of ICE lysate (35S-pro-IL-1β) or 4 μl of CPP32 (35S-PARP) for 1 h at 37 °C. The cleavage products were detected by SDS-polyacrylamide gel electrophoresis and autoradiography as detailed under “Experimental Procedures.” The molecular mass of markers in kDa is indicated at the left of each panel.)
shown). In contrast, an amount of CPP32 (25.7 μg of lysate) that completely cleaves S-PARP (Fig. 4B) partially cleaves α-fodrin (lane 7); the use excess CPP32 (109.3 μg of lysate) resulted in >95% proteolysis of the intact 240-kDa α-fodrin (lane 8). CPP32 cleavage of α-fodrin in vitro produced fragments of 150 kDa (partly obscured by the background 150-kDa fragment) and 120 kDa. These in vitro cleavage fragments were indistinguishable electrophoretically from the in vivo cleavage fragments seen in Jurkat cells treated with anti-Fas mAb (lane 2). Preincubation of CPP32 for 15 min with either 100 nM DEVD-CHO or 50 μM YVAD-CMK completely inhibited α-fodrin proteolysis (data not shown). However, given the contaminating proteases in the purified fodrin preparation, we cannot exclude the possibility that CPP32 is cleaving fodrin indirectly by activating one of these proteases. These in vitro results can be reconciled with the in vivo characterization of the fodrin protease as an ICE/Ced-3 family protease only partially sensitive to inhibition by DEVD-CHO in the following manner. (i) Fodrin proteolysis in vivo may reflect the activity of multiple ICE/Ced-3 family members, including CPP32 and/or CPP32-like proteases; or (ii) the fodrin protease may be an ICE/Ced-3 family member that is less sensitive than CPP32 to DEVD-CHO inhibition.

**DISCUSSION**

We have demonstrated that α-fodrin, an abundant cytoskeletal protein, is cleaved specifically and rapidly during the execution of Fas- and TNF-induced apoptosis, and this cleavage is mediated by an ICE/Ced-3 cysteine protease(s). Both fodrin and PARP proteolysis in vivo during apoptosis are sensitive to inhibition by the specific ICE/Ced-3 protease inhibitors YVAD-CMK, a tetrapeptide inhibitor modeled after the P1–P4 cleavage sequence in pro-IL-1β (1), and by CrmA, a cowpox serpin that selectively inhibits these proteases (20). However, apoptotic fodrin and PARP proteolysis can be distinguished by their dramatically different sensitivities to DEVD-CHO, a tetrapeptide inhibitor identical to the P1–P4 cleavage sequence in PARP that preferentially inhibits CPP32 and CPP32-like ICE/Ced-3 proteases (8, 13, 15). Fodrin proteolysis is relatively insensitive to DEVD-CHO (250 μM concentrations only partially inhibit α-fodrin cleavage in vivo), while PARP cleavage is sensitive to this inhibitor (50 μM concentrations completely prevent PARP proteolysis in vivo). These findings indicate that multiple ICE/Ced-3 proteases are activated during apoptosis, and that these proteases are likely to be acting on a subset of at least partly distinct substrates. Given their potential substrate selectivity, the action of multiple ICE/Ced-3 proteases would be required for the execution of programmed cell death.

Earlier work demonstrating α-fodrin proteolysis during apoptosis induced by anti-Fas mAb and other agents had implicated calpain I, a calcium-activated cysteine protease unrelated to ICE/Ced-3 proteases, as the fodrin protease (44). This conclusion was based largely on indirect evidence, namely the ability of a peptide antibody generated against the N-terminal sequence (GMMPR) of the calpain I cleavage site in α-fodrin to detect the α-fodrin cleavage fragments in apoptotic cells (44). However, this peptide antibody also detected several bands in control, untreated cells. In the present study, Fas- and TNF-induced α-fodrin proteolysis was prevented by specific ICE/Ced-3 inhibitors, but not by the cell-penetrating calpain inhibitors LLM and LLnL, nor by the cysteine protease inhibitors E-64 and leupeptin; these latter inhibitors were used in concentrations previously demonstrated to inhibit calpain activity in vivo (49, 50). Moreover, Jurkat cells stably transfected with a calpastatin cDNA, a gene that specifically inhibits calpains (51), are not resistant to Fas-mediated α-fodrin proteolysis or apoptosis. Taken together, these results indicate that Fas- and TNF-induced α-fodrin proteolysis is mediated by an ICE/Ced-3 protease, not calpain I. Of course, we cannot exclude the possibility that other apoptotic signals activate non-ICE/Ced-3 proteases, including calpains, that subsequently cleave α-fodrin, e.g. calpain I-induced α-fodrin cleavage has been documented by hypoxia (49, 50) and sustained NMDA receptor stimulation (52, 53). More recently, fodrin proteolysis was demonstrated in a cell-free cytosolic extract following the addition of purified granzyme B (54), a serine protease implicated in Fas-independent cytotoxic lymphocyte killing of target cells (55). In contrast to our findings, fodrin proteolysis in this system was inhibited by 0.5 μM DEVD-CHO (54); this discrepancy may reflect differences in the stimuli used to induce apoptosis (anti-Fas mAb and TNFα versus granzyme B) or in the experimental design (in vivo versus cell-free).

Since our in vivo inhibitor studies suggested that the fodrin protease itself, or an upstream activator of this protease, was a relatively DEVD-CHO-insensitive member (or members) of the ICE/Ced-3 family, we examined the ability of bacterially expressed ICE/Ced-3 proteases to cleave purified bovine fodrin in vitro. To our initial surprise, CPP32, a DEVD-CHO-inhibitable protease, cleaved α-fodrin into the identical size fragments observed in apoptotic cells in vivo. In contrast, neither excess ICE nor ICH-1 resulted in any detectable cleavage. Interestingly, α-fodrin contains several potential ICE/Ced-3 protease cleavage sites, including one (DETD 1185-S 1186) (56) just 9 amino acids C-terminal from its calpain I cleavage site determined in vivo (57) and another (DSLD 1478-S 1479) (56) that would yield cleavage fragments of approximately 150 and 120 kDa, the size of the in vivo apoptotic cleavage fragments. These findings suggest that that the fodrin protease itself is an ICE/Ced-3 member; however, definitive proof of this point will require demonstrating that fodrin is indeed cleaved at an aspartic acid residue(s). Moreover, these in vitro results can be reconciled with the in vivo characteristics of the fodrin protease in the following manner. (i) α-Fodrin proteolysis in vivo may reflect the activity of multiple ICE/Ced-3 proteases, including CPP32, that are collectively inhibited by YVAD-CMK and CrmA, but only partially inhibited by DEVD-CHO (reflecting the limited contribution of CPP32); or (ii) the fodrin protease may be an ICE/Ced-3 protease (e.g. a CPP32-like member) that is less sensitive to inhibition by DEVD-CHO.

In addition to defining the enzymatic characteristics of the fodrin protease, the protease inhibitors used in this study have revealed that α-fodrin proteolysis, under certain circumstances, can be uncoupled from cell death. The specific ICE/Ced-3 protease inhibitors YVAD-CMK and CrmA, which are sensitive protease inhibitors TLCK and TPCK prevent Fas- or TNF-induced α-fodrin proteolysis; these inhibitors also block the membrane blebbing and internucleosomal DNA fragmentation characteristic of apoptosis. In contrast to YVAD-CMK and CrmA, TLCK and TPCK are intrinsically toxic and induce a necrotic type of cell death, thereby accounting for the diminished viability of Fas-treated Jurkat cells pretreated with these serine protease inhibitors. The observation that both PARP and α-fodrin remain intact in cells undergoing necrosis rather than apoptosis suggests that cleavage of these substrates may indeed be a specific molecular feature of apoptotic cell death. However, our studies with DEVD-CHO indicate that fodrin cleavage can be observed in cells in the absence of other manifestations of apoptosis. Specifically, 250 μM DEVD-CHO inhibited Fas-induced membrane blebbing, DNA fragmentation, cell death, and PARP cleavage, but only minimally pre-

3 V. Cryns and J. Yuan, unpublished data.
vented fodrin proteolysis (>50% cleaved). Under these circumstances, fodrin proteolysis can be uncoupled from apoptosis, indicating that cleavage of this protein is not sufficient to induce apoptosis. Furthermore, these findings suggest that the fodrin protease is acting either upstream or parallel to the PARP protease during apoptotic cell death. We also observed that PARP proteolysis could be uncoupled from apoptosis. Concentrations of DEVD-CHO (50 μM) that completely prevented PARP cleavage were not sufficient to provide maximal protection against Fas-induced cell death and DNA fragmentation, again suggesting that additional ICE/Ced-3 proteases, other than the PARP protease, also play an important role in the execution of apoptosis.

What is the functional significance of α-fodrin proteolysis during apoptosis? Fodrin (non-erythroid spectrin) is an abundant, highly conserved, spectrin-like protein composed of heterodimers (α- and β-subunits), which self-assemble into tetramers. These tetramers, which are often asymmetrically distributed, are anchored to the plasma-membrane by an ankyrin-like protein and bind to actin, calmodulin, and microtubules. Based on these features, fodrin is thought to play a central role in the dynamic organization of membrane domains and in membrane trafficking events (58). Thus, it is tempting to speculate, as others (44) have done, that fodrin proteolysis during programmed cell death might contribute to the membrane-associated morphological features characteristic of this process, e.g. membrane protrusions/bulging, cytoplasmic shrinkage, and the “rounding up” of cells. In order to delineate the potential role of fodrin cleavage in these events, it will be necessary to develop specific inhibitors of the fodrin protease.

To this end, studies are currently under way to determine the apoptotic cleavage sequences in α-fodrin so that specific peptide inhibitors of this protease can be constructed. Such studies, together with investigations aimed at identifying and characterizing additional cytoskeletal substrates cleaved during cell death, may yield novel insights into the molecular mechanisms of apoptotic cell death.

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