

Simultaneous Degradation of α II- and β II-Spectrin by Caspase 3 (CPP32) in Apoptotic Cells*

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The degradation of α II- and β II-spectrin during apoptosis in cultured human neuroblastoma SH-SY5Y cells was investigated. Immunofluorescent staining showed that the collapse of the cortical spectrin cytoskeleton is an early event following staurosporine challenge. This collapse correlated with the generation of a series of prominent spectrin breakdown products (BDPs) derived from both α II- and β II-subunits. Major C-terminal α II-spectrin BDPs were detected at \approx 150, 145, and 120 kDa (α II-BDP150, α II-BDP145, and α II-BDP120, respectively); major C-terminal β II-spectrin BDPs were at \approx 110 and 85 kDa (β II-BDP110 and β II-BDP85, respectively). N-terminal sequencing of the major fragments produced *in vitro* by caspase 3 revealed that α II-BDP150 and α II-BDP120 were generated by cleavages at DETD¹¹⁸⁵*S¹¹⁸⁶ and DSLD¹⁴⁷⁸*S¹⁴⁷⁹, respectively. For β II-spectrin, a major caspase site was detected at DEVD¹⁴⁵⁷*S¹⁴⁵⁸, and both β II-BDP110 and β II-BDP85 shared a common N-terminal sequence starting with Ser¹⁴⁵⁸. An additional cleavage site near the C terminus, at ETVD²¹⁴⁶*S²¹⁴⁷, was found to account for β II-BDP85. Studies using specific caspase or calpain inhibitors indicate that the pattern of spectrin breakdown during apoptosis differs from that during non-apoptotic cell death. We postulate that in concert with calpain, caspase rapidly targets critical sites in both α II- and β II-spectrin and thereby initiates a rapid dissolution of the spectrin-actin cortical cytoskeleton with apoptosis.

The importance of proteases in the expression of mammalian apoptosis has been the subject of many recent studies. The mammalian interleukin-1 β -converting enzyme (ICE)¹-like pro-

tease family (renamed caspase (1)) is perhaps the best characterized. Overexpression of ICE in fibroblasts can lead to apoptosis (2). While at least eight other caspases have been identified (Ich-1 (Nedd2), ICE-LAP6, Ich-2, ICERelIII, Mch-2, Mch-3, Mch-4, and Mch-5/FLICE (for reviews, see Refs. 3 and 4)), human caspase 3 (CPP32) is perhaps the most universal apoptosis mediator. It is present in most mammalian cells (5), and its deletion by gene knockout blocks neuronal death during brain development with consequential lethality (6). Besides the caspases, a second family of proteases implicated in the initiation and control of apoptosis are the calpains (7, 8), especially in several hematopoietic and neuronal cells (9–12). The relationship between these two protease families, the consequences of each on their respective substrates and on cellular physiology, or the conditions under which each is activated remain poorly understood.

While many proteins are cleaved during apoptosis, a prominent target of both calpain and caspase action is α II-spectrin, the major component of the cortical membrane skeleton. In neurons, calcium-activated calpain cleavage of α II-spectrin (non-erythroid α -spectrin or α -fodrin) accompanies N-methyl-D-aspartic acid receptor activation (13),² does not directly cause neuronal toxicity (7, 15), and is postulated to be necessary for synaptic and neuronal plasticity (16–18). Indeed, α II-spectrin cleavage by calpain appears to be a molecular mechanism by which skeletal plasticity can be enhanced without complete dissolution of the spectrin skeleton since calpain-mediated cleavage of α II-spectrin bestows calmodulin regulation on oligomeric spectrin-actin complexes, but does not dissociate them (unless β II-spectrin is also cleaved) (19, 20). In addition to the action of calpain, α II-spectrin is also targeted by caspase 3 during apoptosis in lymphocytes, hematopoietic cells, and neurons (12, 21–25). A central question is to understand the molecular consequences of each protease's action, both singly and in concert, on spectrin's many functions and on the integrity of the cortical spectrin skeleton, a structure required for the maintenance of membrane order and integrity (reviewed in Ref. 26).

In other work, we have demonstrated that beyond the specific and preferred site of calpain action at the Tyr¹¹⁷⁶–Gly¹¹⁷⁷ bond (VY*GMMPR) in α II-spectrin (27), calpain also targets several additional sites in both α II- and β II-spectrin (27).² In the present report, we demonstrate the specific sites of caspase 3 cleavage within both α II- and β II-spectrin and show that during apoptotic induction in neuroblastoma SH-SY5Y cells, it is caspase 3 that most rapidly cleaves not only α II-spectrin, but

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¹ The abbreviations used are: ICE, interleukin-1 β -converting enzyme; MTX, maitotoxin; Z-D-DCB, benzyloxycarbonyl-Asp-CH₂OC(O)-2,6-dichlorobenzene; GST, glutathione S-transferase from *Schistosoma japonicum*; PAGE, polyacrylamide gel electrophoresis; pAb, polyclonal antibody; CAPS, 3-(cyclohexylamino)propanesulfonic acid; BDP, breakdown product; α II-BDP, α II-spectrin breakdown product; β II-BDP, β II-spectrin breakdown product; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

² S. P. Glantz, C. D. Cianci, K. K. W. Wang, and J. S. Morrow, submitted for publication.

also β II-spectrin, and that this process is accompanied by skeletal dissolution. Together, these results define the molecular targets of these two important protease systems on the spectrin skeleton and suggest a mechanism by which different proteases, acting at slightly different sites within spectrin, might alternatively induce either enhanced skeletal plasticity or membrane skeletal dissolution.

MATERIALS AND METHODS

MTX and Staurosporine Treatment of SH-SY5Y Cells—Human neuroblastoma SH-SY5Y cells (SY5Y) were grown to confluence on 12-well plates ($\sim 2 \times 10^6$ cells/well) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2.5 μ g/ml Fungizone (amphotericin B). Cultures were washed three times with serum-free minimum essential medium. After 1 h of preincubation with calpain inhibitor I (acetyl-Leu-Leu-Nle-CHO, Calbiochem) or Z-D-DCB (a caspase inhibitor), cultures were challenged either with a 0.1 nM concentration of the calcium channel activator maitotoxin (28) or with 0.5 μ M staurosporine, each for 1 h (29). Unless otherwise stated, cultures were washed and returned to regular non-serum medium for 24 h, when cell viability was monitored or protein or DNA extraction was performed.

In Vitro Spectrin and GST Fusion Peptide Digestion—Purified heteromeric bovine brain spectrin² was digested with purified recombinant caspases (30, 31) or purified porcine μ -calpain or m-calpain in 100 mM Hepes (pH 7.2 at 25 °C), 10 mM dithiothreitol, 10% (v/v) glycerol, and 1 mM EGTA for 90 min. Digestion was halted by the addition of an equal volume of SDS-PAGE sample buffer. Triplicate samples were subjected to electrophoresis. One gel was stained with Coomassie Blue, whereas two were transferred to Immobilon[®] membranes and probed by Western blotting with anti- α II-spectrin (pAb RAF-A) and anti- β II-spectrin (pAb 10D) antibodies, respectively.² Similar experiments were also carried out on GST fusion peptides representing various regions of α II- and β II-spectrins, prepared and analyzed as described previously (32).² GST-containing fusion peptides were also detected by Western blotting using anti-GST antibody (Amersham Pharmacia Biotech).

N-terminal Sequence Determinations—After SDS-PAGE on either 8% polyacrylamide or 4–20% gradient gels, samples were transferred to polyvinylidene difluoride membranes using 20 mM CAPS and 10% methanol (pH 11.0) for 60–75 min. The membrane was rinsed with water, stained with 0.5% Coomassie Blue in 50% methanol, and destained briefly with 100% methanol. Upon drying, the bands of interest were cut from the blot and subjected to N-terminal determination using an Applied Biosystems protein sequencer.

Cell Death Measurement—SY5Y cell death was assessed by measuring the cytosolic enzyme lactate dehydrogenase released into the medium (25- μ l samples) (39). Alternatively, 40 μ g/ml propidium iodide was added directly to the cell culture wells at 24 h, and fluorescence of the dye-DNA complex (excitation at 530 nm and emission at 620 nm) was measured after 5 min with a Millipore Cytofluor 2300 fluorescence plate reader.

Protein Extraction and Analysis—At the end of an experiment, the medium was first removed, and the attached cells were washed twice with Tris-buffered saline and 1 mM EDTA. Protein extraction was accomplished by cell lysis with SDS, followed by protein precipitation with trichloroacetic acid and solubilization with Tris base (33). Protein samples were analyzed for protein concentration with a modified Lowry assay (Bio-Rad). An equal amount of total protein (15 μ g) was loaded onto each lane and run on SDS-polyacrylamide gel (4–20% acrylamide) with a Tris/glycine running buffer system and then transferred to polyvinylidene difluoride membrane (0.2 μ m) using a Tris/glycine buffer system in a semidry electrotransfer unit (Bio-Rad) at 20 mA for 1.5–2 h. α II-Spectrin was detected with monoclonal antibody 1622 (Chemicon International, Inc.), pAb RAF-A, or pAb RAF-B (34). β II-Spectrin was detected by pAb 10D, raised against a recombinant β II-spectrin peptide representing repeat unit 13 to residue 2204 within domain III (β II_{13-C3}) (32). Biotinylated second antibody and avidin conjugated to alkaline phosphatase were from Amersham Pharmacia Biotech. The blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Densitometric analysis of Western blots was performed using a color scanner (Umax UC630) and either the National Institutes of Health program Image 1.5 or the program ScanAnalysis (Biosoft) operating on an Apple Macintosh[®] computer. Nonlinear regression analysis was carried out using the Biosoft program Ultrafit (Version 3.0), also operating on a Macintosh[®] computer.

Immunofluorescence Studies—Immunofluorescence studies em-

ployed SH-SY5Y cells cultured on German glass coverslips (Fisher) at medium density. At the desired time point upon staurosporine treatment (6 h, unless stated otherwise), cells from all experimental treatment groups were fixed in 4% paraformaldehyde for 2 h at room temperature. Following fixation, cells were washed twice with phosphate-buffered saline (136 mM NaCl, 81 mM KCl, 1.6 mM Na₂HPO₄, and 14 mM KH₂PO₄ (pH 7.4)). Cultures were first incubated in 5% nonfat dry milk at 4 °C for 2 h. Primary antibodies (anti-CPP32 and anti-poly(ADP-ribose)polymerase) in blocking solution (10 mM NaPO₄ (pH 7.5), 0.9% NaCl, 0.1% Tween 20, and 5% nonfat dry milk) were incubated for 3 h at 25 °C. The cultures were then washed with blocking solution three times for 10 min. Secondary antibodies (anti-mouse IgG) linked to a specific fluorophore (fluorescein isothiocyanate) were applied for 2 h. The coverslips were then washed three times in phosphate-buffered saline solution, mounted on slides containing Elvanol (anti-fade agent, DuPont), and allowed to dry. Slides were stored in the dark prior to immunofluorescence microscopic examination. Control cultures without primary antibodies did not stain.

RESULTS

Both α II- and β II-Spectrin Are Degraded in Apoptotic SY5Y Cells—Our previous studies have established that SY5Y cells subjected to staurosporine (0.5 μ M) undergo apoptosis, with chromatin condensation and other stigmata of apoptosis appearing within 5–6 h. The number of cells dying in culture plateau within 16–24 h (12, 35). In the current study, loss of intact α II- and β II-spectrin in staurosporine-treated cells was detected by 1 h and continued progressively for 24 h (Fig. 1A). This loss was mirrored by increased levels of α II-spectrin BDPs at \approx 150, 145, and 120 kDa (α II-BDP150, α II-BDP145, and α II-BDP120, respectively). β II-Spectrin was also simultaneously degraded into two major immunoreactive fragments at \approx 110 and 85 kDa (β II-BDP110 and β II-BDP85, respectively). A minor β II-BDP was also evident at \approx 55 kDa (β II-BDP55) (Fig. 1B).

The time course of the breakdown of both α II- and β II-spectrins into their major cleavage fragments could be roughly modeled as a three-step kinetic process, in which each intact subunit (A) broke down to an intermediate product (B) and then was then further degraded to a smaller major product (C), with two corresponding rate constants, k_A and k_B (Equation 1).



Scaling data to unit maximum value for A (the starting value of α II- or β II-spectrin) and solving the resulting kinetic equation as a function of time (t) yield the relative amount of B or C (Equations 2 and 3).

$$B = \frac{k_A(e^{-k_A t} - e^{-k_B t})}{(k_B - k_A)} \quad (\text{Eq. 2})$$

$$C = (1 - e^{-k_A t}) - \frac{k_A(e^{-k_A t} - e^{-k_B t})}{(k_B - k_A)} \quad (\text{Eq. 3})$$

Fitting the observed breakdown patterns using Equations 2 and 3 indicated that $k_A \approx 0.17 \text{ h}^{-1}$ (for generation of α II-BDP150) and that $k_B \approx 0.12 \text{ h}^{-1}$ (for generation of α II-BDP120) (Fig. 1B). For β II-spectrin, the corresponding values were $k_A \approx 0.16 \text{ h}^{-1}$ (for generation of β II-BDP110) and $k_B \approx 0.03 \text{ h}^{-1}$ (for generation of β II-BDP85). These rates correspond to half-lives ($t_{1/2}$) for intact α II-spectrin of $\approx 2.9 \text{ h}$ and for β II-spectrin of $\approx 3.1 \text{ h}$. The half-lives of the intermediates derived from these fits were $\approx 4.2 \text{ h}$ for α II-BDP150 and $\approx 19.2 \text{ h}$ for β II-BDP110. Although these $t_{1/2}$ values tended to vary ($\pm 0.8 \text{ h}$) from experiment to experiment (data not shown), it was clear that unlike for calpain cleavage (19),² the time course of β II-spectrin breakdown by caspase 3 essentially paralleled that of α II-spectrin.

Except for the transient generation of α II-BDP150, which can arise from proteolysis by several different proteases at a

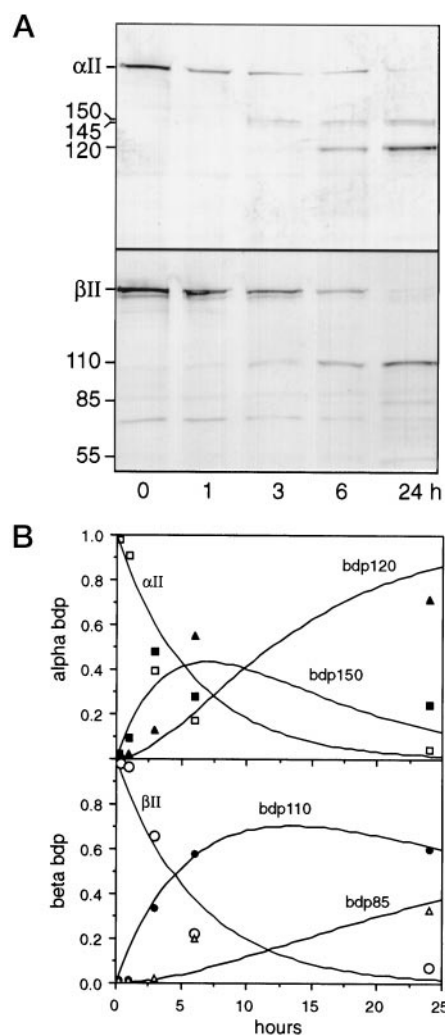


FIG. 1. Time course of α II- and β II-spectrin breakdown in apoptotic SY5Y cells. A, SH-SY5Y cells were challenged with $0.5 \mu\text{M}$ staurosporine for 0–24 h. Total cellular protein was extracted and analyzed ($15 \mu\text{g}/\text{lane}$) by SDS-PAGE and Western blotting using monoclonal antibody 1622 (anti- α II-spectrin) or pAb 10D (anti- β II-spectrin). The position of intact α II-spectrin (upper panel) or β II-spectrin (lower panel) is marked, as are the apparent sizes (in kDa) of the major immunoreactive BDPs. Results shown are representative of at least three experiments. B, shown are the results from the quantitative analysis of the time course of spectrin breakdown during apoptosis. The abscissa represents time after exposure to staurosporine. Relative levels of α II-spectrin (open squares), α II-BDP150 (closed squares), α II-BDP120 (closed triangles), β II-spectrin (open circles), β II-BDP110 (closed circles), and β II-BDP85 (open triangles) are plotted. Data were fitted by nonlinear regression simultaneously to the three-state model $A \rightarrow B \rightarrow C$, according to Equations 2 and 3 (see “Results”). The mean square deviations of the fits were $R^2 = 0.2452$ for α II-spectrin and 0.1269 for β II-spectrin.

hypersensitive region within α II-spectrin repeat unit 11 (27, 36),² the pattern of BDPs generated by staurosporine action in the SY5Y cells appeared to be distinct from those produced by calpain.² To further define the nature of the protease cascade operating in these experiments, the effects of specific calpain and caspase inhibitors on staurosporine-induced breakdown were compared (Fig. 2). Calpain inhibitor I specifically blocked the generation of α II-BDP145, but did not affect the generation of the major product, α II-BDP120 (Fig. 2A). Conversely, Z-D-DCB, a caspase inhibitor, blocked the appearance of α II-BDP120. Both inhibitors slowed the overall generation of α II-BDPs, and thus, it appeared that both proteases contributed in some measure to the generation of α II-spectrin BDPs. With respect to β II-spectrin, calpain inhibitor I had little effect on

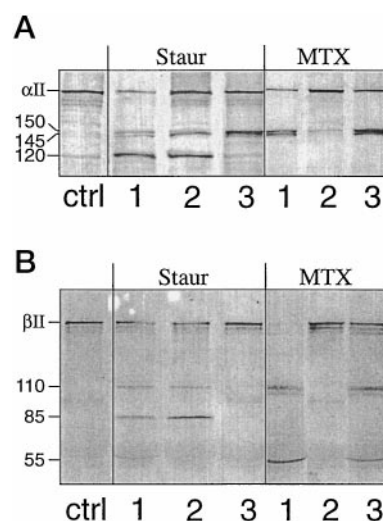


FIG. 2. Staurosporine-induced apoptosis in SY5Y cells is mainly caspase-mediated. Shown are the effects of caspase and calpain inhibitors on α II-spectrin (A) and β II-spectrin (B) breakdown in SY5Y cells. Cells were either untreated (ctrl) or challenged with $0.5 \mu\text{M}$ staurosporine (Staur) for 5 h or with 0.01 nM MTX for 3 h. Staurosporine initiates apoptosis in these cells, whereas MTX induces non-apoptotic cell death. These experiments were carried out in the presence of no inhibitor (lane 1), $10 \mu\text{M}$ calpain inhibitor I (lane 2), or $50 \mu\text{M}$ Z-D-DCB (a caspase inhibitor) (lane 3). After SDS-PAGE, Western blots were probed with either pAb RAF-A (A) or pAb 10D (B). The positions of the intact subunits and the sizes of major BDPs are as indicated. Results shown are representative of at least three experiments.

β II-spectrin degradation compared with Z-D-DCB, which blocked the cleavage of β II-spectrin almost completely (Fig. 2B). These patterns of cleavage were distinct from those induced in SY5Y cells by treatment with MTX (0.01 nM), a highly potent marine toxin that activates both voltage-sensitive and receptor-operated calcium channels in the plasma membrane (Fig. 2). Presumably, the intracellular calcium load induced by such treatment activates cell death pathways similar to those operating during necrosis (7, 28). With MTX treatment, there was dramatic loss of both α II- and β II-spectrin coupled with the generation of α II-BDP150, β II-BDP110, and β II-BDP55. On the other hand, the α II-BDP120 and β II-BDP85 products, characteristic of caspase 3 activity, were not formed. Z-D-DCB provided minimal protection, whereas calpain inhibitor I almost completely blocked both α II- and β II-spectrin breakdown in MTX-treated cells (Fig. 2). A β II-spectrin cleavage product of $\approx 110 \text{ kDa}$ observed with MTX is similar in size to the caspase-generated β II-BDP110; based on the lack of β II-BDP85 as well as the inhibition of this cleavage by calpain inhibitor I, it appears that this band is a calpain product. These data, together with our earlier studies (12, 28), indicate that staurosporine and MTX activate in large measure distinct pathways of spectrin proteolytic cleavage in SY5Y cells. These pathways appear to be characteristic of apoptotic and non-apoptotic (necrotic) cell death, respectively, and both involve cleavage of β II-spectrin as part of the cell death event.

α II/ β II-Spectrin Is Digested Most Readily by Caspase 3—The pattern of spectrin degradation during staurosporine-induced apoptosis in SY5Y cells and the response of these cells to Z-D-DCB strongly implicated a caspase in the apoptotic breakdown of spectrin. Since several related caspases may be active during apoptosis (37), it was of interest to determine their relative activity against spectrin in the milieu of SY5Y cells. Cell lysates were thus digested for 1 h with comparable amounts of recombinant human caspases 1–4, 6, and 7, and the breakdown patterns were analyzed after Western blotting (Fig. 3). As expected, all caspases generated α II-BDP150, presum-

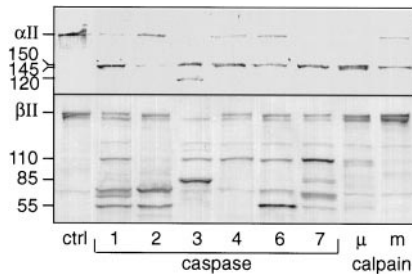


FIG. 3. Different caspases yield distinct spectrin cleavage patterns. SY5Y cellular extracts (20 μ g of protein) were subjected to *in vitro* digestion with 0.5 μ g each of caspases 1–4 and 6–8 for 2 h. Alternatively, the same extracts were digested with 0.5 μ g of μ -calpain or m-calpain for 30 min at ambient temperature or were sham-digested without enzyme (*ctrl*). Samples were analyzed by SDS-PAGE and Western blotting with pAb RAF-A or pAb 10D. The positions of the intact protein and major cleavage products for α II-spectrin (*upper panel*) and β II-spectrin (*lower panel*) are as indicated (in kDa). Results shown are representative of three experiments.

ably due to cleavage within α II-spectrin's hypersensitive site. In contrast, only caspase 3 produced significant levels of α II-BDP120. All caspases also readily digested β II-spectrin, but differed significantly in the ratio of the major β II-spectrin fragments generated (Fig. 3). For example, the dominant products generated by caspases 2, 3, 6, and 7 were β II-BDP70, β II-BDP85, β II-BDP55, and β II-BDP110, respectively. Again, only caspase 3 produced significant levels of both β II-BDP110 and β II-BDP85. While additional β II-spectrin BDPs no doubt existed in these experiments that were not visualized by pAb 10D (which is directed to the C-terminal third of β II-spectrin), these findings establish that caspases display characteristic differences in their relative specificity and activity *vis-à-vis* spectrin. Variations in the amount of active enzyme or in the enzyme/substrate ratios are unlikely to be a factor in these experiments since even in the very same experiment (*e.g.* with caspase 2; Fig. 3), a protease that displayed minimal activity against α II-spectrin often showed the greatest activity against β II-spectrin. Interestingly, α II-BDP120, β II-BDP110, and β II-BDP85, the spectrin fragments most prominent in apoptotic SY5Y cells (*cf.* Fig. 1), are characteristic of caspase 3 action (Fig. 3). Conversely, neither μ -calpain nor m-calpain, even when added to the SY5Y lysates, generated these fragments. Taken together, these data indicate that caspase 3 is the dominant protease mediating spectrin cleavage in staurosporine-induced apoptosis in neuroblastoma SY5Y cells.

Caspase 3 Cleaves Both α II- and β II-Spectrin at Multiple Consensus Recognition Sites—The sites of calpain cleavage in both α II- and β II-spectrin have been identified (27).² To identify the precise sites at which caspase 3 cleaves spectrin, purified bovine brain α II/ β II-spectrin was digested *in vitro*, and the resulting digestion products were analyzed by Western blotting and N-terminal microsequencing. Caspase 3 generated multiple spectrin fragments ranging from \approx 165 to \approx 85 kDa on Coomassie Blue-stained gels (Fig. 4). Two of these products (α II-BDP150 and α II-BDP120) reacted with monoclonal antibody 1622, indicating their origin from the α II-subunit. Conversely, pAb 10D detected β II-fragments at \approx 110, \approx 100, \approx 85, and \approx 55 kDa. Only the Coomassie Blue-stained fragment at \approx 165 kDa was unaccounted for on the Western blots; presumably this represents a β II-spectrin N-terminal fragment that was not detected by pAb 10D. Collectively, the *in vitro* caspase 3 digestion products were almost identical to those observed in staurosporine-induced SY5Y cells (except for the α II-BDP145 generated by calpain).

N-terminal sequencing identified the origin of several major caspase 3-generated fragments. Due to the presence of multiple

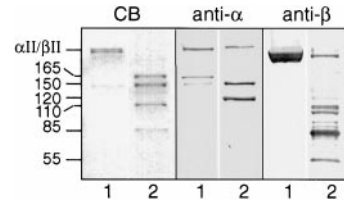


FIG. 4. *In vitro* digestion of α II/ β II-spectrin by caspase 3. Bovine α II/ β II-spectrin heterotetramer (20 μ g) was digested with 1 μ g of caspase 3 (CPP32, beta form) for 1 h at 25 $^{\circ}$ C. The samples were analyzed by PAGE followed by Coomassie Blue staining (*CB*) or probed with antibody against α II-spectrin (*anti- α*) or β II-spectrin (*anti- β*). Undigested material is in *lane 1*, and digested material is in *lane 2*. The intact α II- and β II-spectrin subunits and the apparent sizes (in kDa) of their major fragments are indicated. Results shown are representative of three experiments. Note the appearance of the \approx 165-kDa band, which arises from the N-terminal half of β II-spectrin. pAb 10D does not react with this portion of β II-spectrin.

bands of very similar molecular mass, the identity of some fragments proved difficult to obtain. These results (in which at least six terminal residues could be determined with confidence) are summarized in Table I and in Fig. 8. The α II-BDP150 fragment mapped to the sequence DETD¹¹⁸⁵*S¹¹⁸⁶KTASP in repeat 11 (with * representing the site of cleavage and the beginning point of the determined sequences). This site is just distal to the major calpain cleavage site (VY¹¹⁷⁶*G¹¹⁷⁷MMP) and immediately proximal to the calmodulin-binding domain (residues 1187–1206) (27). It is also likely that the N-terminal half of α II-spectrin was present within the α II-BDP150 band, based on analogy with the cleavage of α II-spectrin by calpain (7, 36).² However, given the blocked N terminus of α II-spectrin, this fragment did not appear in the microsequencing results. The α II-BDP120 fragment mapped to a second caspase 3 cleavage site (DSL¹⁴⁷⁸*S¹⁴⁷⁹EALIKKHE) in repeat 14 of α II-spectrin. The fragment liberated from α II-BDP150 to yield α II-BDP120 appeared in α II-BDP35 (Table I). Both the β II-BDP110 and β II-BDP85 fragments shared a common N-terminal sequence, placing this site of cleavage in repeat 11 of β II-spectrin (DEV¹⁴⁵⁷*S¹⁴⁵⁸KRLTVQT). Reliable sequence information was not obtained from β II-BDP55 due to its low abundance.

Additional studies were carried out on a series of recombinant GST-spectrin fusion polypeptides to validate the above assignments and to assess whether the quaternary structure of the spectrin heterodimer modifies its susceptibility to caspase 3 (as it does with calpain) (19).² GST fusion proteins encompassing three overlapping segments of α II-spectrin (from repeat 9 through the C terminus) were digested with caspase 3 (Fig. 5). The GST- α II_{9–12} polypeptide was cleaved by caspase 3 into fragments of 75 and 20 kDa. Autolytic fragments of caspase 3 were also evident in these gels (Fig. 5A, *arrows*). The anti-GST antibodies reacted with the 75-kDa fragment, indicating its origin from the N-terminal half of the fusion peptide (Fig. 5B). End sequencing of the 20-kDa fragment yielded -SKTASPWK-SAR, identical to the cleavage site generating α II-BDP150 from caspase treatment of the intact heterotetramer (Table I). Similarly, digestion of GST- α II_{13–18} generated two fragments of \approx 50 and \approx 45 kDa (Fig. 5A). Antibodies against GST and α II-spectrin (monoclonal antibody 1622) reacted with the 45- and 50-kDa fragments, respectively, indicating that they represented the respective N- and C-terminal halves of the fusion polypeptide (Fig. 5, B and C). The N-terminal sequence of the 50-kDa fragment was -SVEALIKKH, identical to the α II-BDP120 cleavage site (Table I). Finally, the GST- α II_{18–C} polypeptide resisted digestion with caspase 3.

Two GST fusion polypeptides representing regions of β II-spectrin were also subjected to caspase 3 digestion (Fig. 6). GST- β II_{8–13} extended from repeat 8 through repeat 13, and GST- β II_{13–C Δ} from repeat 13 to residue 2204 within the C-terminal domain III (32).

TABLE I
Major caspase 3-generated spectrin fragments

All digestions were carried out *in vitro*. Sequence numbering was based on α II Σ 1-spectrin (GenBank™ U83867) (footnote 3) and β II-spectrin (GenBank™ M96803) (53). The sequence of GST26 was from *S. japonicum* (42).

Fragment	N-terminal sequence	Predicted cleavage site	Assumed end	Calculated M_r
Native protein				
α II-BDP150' ^a	(MDPSGVKVLK)	(Start of α II-spectrin, assumed)	Asp ¹¹⁸⁵	136,833
α II-BDP150	-SKTAXPWKXA	DETD ¹¹⁸⁵ *S ¹¹⁸⁶ KTASPWKSA	Asn ²⁴⁷⁷	148,278
α II-BDP120	-SVEALIKKH	DSL ¹⁴⁷⁸ *S ¹⁴⁷⁹ VEALIKKHED	Asn ²⁴⁷⁷	114,602
α II-BDP35	-SKTASPWKSAR	DETD ¹¹⁸⁵ *S ¹¹⁸⁶ KTASPWKSAR	Asp ¹⁴⁷⁸	33,693
β II-BDP165	(MTTIVATD)	(Start of β II-spectrin, assumed)	Asp ¹⁴⁵⁷	170,061
β II-BDP110	-SKRLTVQTKF	DEVD ¹⁴⁵⁷ *S ¹⁴⁵⁸ KRLTVQTKFM	Lys ²³⁶⁴	104,614
β II-BDP85	-SKRLTVQTKF	DEVD ¹⁴⁵⁷ *S ¹⁴⁵⁸ KRLTVQTKF	Asp ²¹⁴⁶	80,404
β II-BDP55	ND ^b			
GST constructs				
GST- α II ₉₋₁₂		(Start of α II-sequence at Glu ⁸³⁷)	Ser ¹²⁶⁷	76,331
75 kDa	-MSPILGYWKI	N terminus of GST	Asp ¹¹⁸⁵	66,818
20 kDa	-SKTASPWXSXA	DETD ¹¹⁸⁵ *S ¹¹⁸⁶ KTASPWKSAR	Ser ¹²⁶⁷	9,536
GST- α II ₁₃₋₁₈		(Start of α II-sequence at Ala ¹³³³)	Lys ¹⁹²⁸	95,113
50 kDa	-SVEALIKKH	DSL ¹⁴⁷⁸ *S ¹⁴⁷⁹ VEALIKKHED	Lys ¹⁹²⁸	51,292
45 kDa	-MSPILGYWKI	N terminus of GST	Asp ¹⁴⁷⁸	43,838
GST- α II _{18-C}	(No BDP detected)	(Start of α II-sequence at position 1929)	Asn ²⁴⁷⁷	90,291
GST- β II ₈₋₁₃		(Start of β II-sequence at Ala ¹²²⁷)	Leu ¹⁶⁵⁸	78,777
48 kDa	-MSPILGYWKI	N terminus of GST	Asp ¹⁴⁵⁷	55,230
22 kDa	-SKRLTVQTKF	DEVD ¹⁴⁵⁷ *S ¹⁴⁵⁸ KRLTVQTKFM	Leu ¹⁶⁵⁸	23,563
21 kDa	-MSPILGYWKI	N terminus of GST	Asp ¹²⁵⁴	31,440
20 kDa	-DRHRKNRETA	DSID ¹²⁵⁴ *D ¹²⁵⁵ RHRKNRETA	Asp ¹⁴⁵⁷	23,808
GST- β II _{13-CA}		(Start of β II-sequence at Val ¹⁶⁷⁶)	Leu ²²⁰⁴	89,333
77 kDa	-MSPILGYWKI	N terminus of GST	Asp ²¹⁴⁶	83,272
8 kDa	-TSEMVNGATE	ETVD ²¹⁴⁶ *T ²¹⁴⁷ SEMVNGATE	Leu ²²⁰⁴	6,192

^a It is likely that a second cleavage product also is present in the α II-BDP150 band, representing α II-spectrin residues 1–1185; this product (α II-BDP150') cannot be detected by end sequencing since the N terminus of spectrin is methylated.

^b ND, not determined.

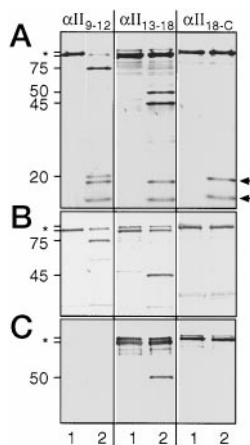


FIG. 5. Digestion of GST- α II-spectrin fusion proteins by caspase 3. GST fusion proteins α II₉₋₁₂, α II₁₃₋₁₈, and α II_{18-C} (15 μ g each) were either left untreated (lane 1) or digested with 0.8 μ g of caspase 3 for 1 h at 25 °C (lane 2). The samples were analyzed by SDS-PAGE followed by staining with Coomassie Blue (A) or probed with antibodies against either GST (B) or α II-spectrin (C). The intact fusion proteins (*) and the apparent sizes (in kDa) of their major digestion fragments are shown. The positions of major autolytic fragments of caspase 3 are indicated by arrows. Results shown are representative of two experiments.

GST- β II₈₋₁₃ (\approx 72 kDa) was rapidly cleaved to an \approx 48-kDa fragment and several bands around 22–20 kDa (Fig. 6A). The 48- and 21-kDa bands were immunoreactive with anti-GST antibody and contained native GST N-terminal sequence (Fig. 6B). The N termini of the 22-kDa fragment and the 20-kDa minor fragment were generated by the cleavages DEVD¹⁴⁵⁷*S¹⁴⁵⁸KRLTVQTKF and DSID¹²⁵⁴*D¹²⁵⁵RHRKNRETA, respectively (Table I). Evaluation of GST- β II_{13-CA} (85 kDa) was complicated by the sensitivity of this peptide to degradation during its expression and preparation (32); consequently, a number of bands were apparent after SDS-PAGE

even in the absence of added caspase 3. Nevertheless, two cleavage products at \approx 77 and \approx 8 kDa were identified as caspase-specific (Fig. 6C). The intact fusion polypeptide and the 77-kDa fragment immunoreacted with anti-GST and 10D antibodies, and both shared an intact GST N-terminal sequence (Table I). The sequence of the 8-kDa fragment, which did not react with either antibody, resulted from cleavage at ETVD²¹⁴⁶*T²¹⁴⁷SEMVNGATE (Table I).

Immunofluorescence Staining—The integrity of the spectrin-based cytoskeleton in apoptotic SY5Y cells was evaluated by indirect immunofluorescence using α II-spectrin-specific (pAb RAF-A) and β II-spectrin-specific (pAb 10D) antibodies. Control cells demonstrated a subplasmalemma distribution of α II/ β II-spectrin, extending to the cell processes (Fig. 7, D and G), consonant with its localization in most other mature cells (26). In 5 h of staurosporine treatment, significant α II- and β II-spectrin breakdown occurred (Fig. 1), and α II-spectrin collapsed into localized cytosolic aggregates coincident with cell shrinkage and fragmentation of the cell processes (Fig. 7E). Cytosolic spectrin was also prominent in dissociated apoptotic bodies. The changes in β II-spectrin paralleled those in α II-spectrin (Fig. 7H). These changes were blocked partially by Z-D-DCB cotreatment (Fig. 7, F and I).

DISCUSSION

Beginning with the observation that α II-spectrin cleavage is tightly coupled to the process of apoptosis in lymphocytes (21), several studies have noted the generation of an \approx 150-kDa BDP of α II-spectrin (12, 22–25). This is perhaps not a surprising observation given the extreme sensitivity of α II-spectrin to a variety of proteases that cleave at or near the junction of repeat units 10–11 (27, 36). Other cleavages have also been occasionally noted, without definitive identification of their origin. The present report significantly extends these observations by establishing (i) that β II-spectrin is cleaved as rapidly as α II-spectrin during staurosporine-induced apoptosis; (ii) that the primary protease acting on spectrin under these conditions is

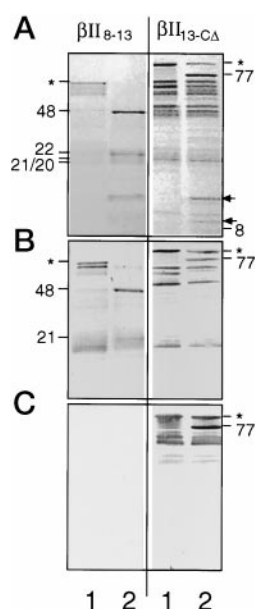


FIG. 6. Digestion of GST- β II-spectrin fusion proteins by caspase 3. GST fusion proteins β II₈₋₁₃ and β II_{13-CA} (15 μ g each) were either left untreated (lane 1) or digested with 0.8 μ g of caspase 3 for 1 h at 25 °C (lane 2). The samples were analyzed by SDS-PAGE followed by staining with Coomassie Blue (A) or probed with antibody against either GST (B) or β II-spectrin (C). As expected, there was no immunoreactivity of the β II₈₋₁₃ peptide or its fragments with the anti- β II-spectrin antibody (pAb 10D) since this antibody does not recognize epitopes upstream of repeat 13. The intact fusion proteins (*) and the apparent sizes (in kDa) of their major digestion fragments are shown. The positions of major autolytic fragments of caspase 3 are indicated by arrows. Results shown are representative of two experiments.

caspase 3; (iii) that the cleavage of β II-spectrin correlates temporally with the onset of apoptosis; and (iv) that caspase 3 cleaves at precise and characteristic sequences within both α II- and β II-spectrins *in vivo* and *in vitro*. These findings place on a firm structural basis our understanding of caspase action on spectrin and, together with data on the distinct actions of calpain (19, 20, 27),² suggest a key and perhaps mechanistic role for β II-spectrin breakdown in the apoptotic process.

The cleavage of spectrin by caspase 3 is interesting in several respects. Four of five cleavage sequences found in native α II- and β II-spectrins (DETD*S, DSLD*S, DEVD*S, and DSID*D) fit the preferred P4-P1 consensus for caspase 3 (38). The serine residue in the P1' position is also found in three sites, as it is in several other caspase substrates (*e.g.* SERBP-2, D4-GDI, and hungtinin) (39–41). It thus appears that the P4-P1' sequence DXXD*S is strongly preferred as an endogenous substrate for caspase 3. However, the heterodimeric unit of spectrin contains 36 DXXD sequence motifs, yet caspase 3 efficiently cleaves α II-spectrin at only two sites and β II-spectrin at three (or possibly four or five) sites (Fig. 8). The time course of these cleavages also suggests that there is a hierarchy in the proteolytic cascade, such that not all potential cleavage sites are simultaneously exposed. The result is that all possible combinations of BDPs do not appear (such as the lack of an α II-spectrin cleavage product representing residues 1–1478, calculated M_r of 170,530). The approximate steps in the spectrin proteolytic cascade initiated by caspase are summarized in Fig. 8. The first cleavage in α II-spectrin occurs between Asp¹¹⁸⁵ and Ser¹¹⁸⁶. This site is just nine residues C-terminal to the initial site of cleavage by calpain and still proximal to the calmodulin-binding domain (27). This cleavage divides α II-spectrin approximately in half. Subsequently, caspase cuts α II-spectrin between D¹⁴⁷⁸*S¹⁴⁷⁹, a site positioned between helices A and B in

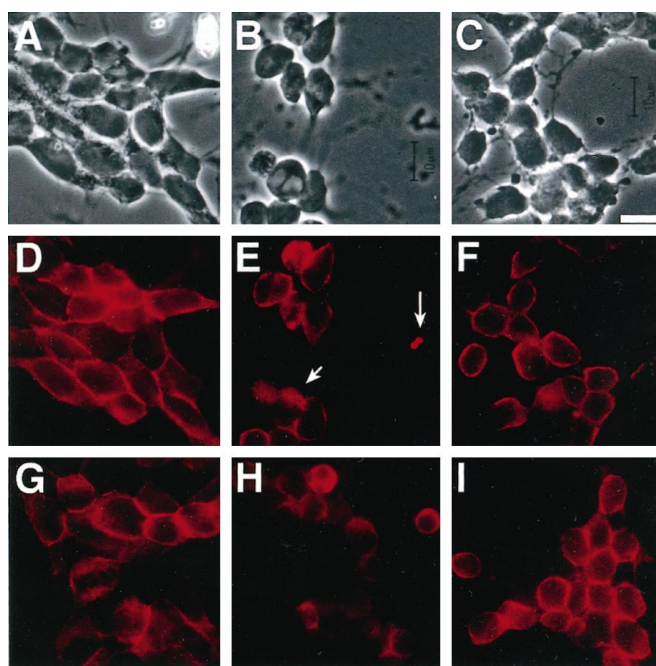


FIG. 7. Immunofluorescence of α II- and β II-spectrins in apoptotic SH-SY5Y cells. SY5Y cells were either untreated (A, D, and G) or challenged with 0.5 μ M staurosporine for 5 h in the absence (B, E, and H) or presence (C, F, and I) of 100 μ M Z-D-DCB. The cells were then fixed and examined by phase microscopy (A–C) or subjected to immunofluorescence analysis with antibodies to α II-spectrin (pAb RAF-A; D–F) and β II-spectrin (pAb 10D; G–I). Note the uniform labeling of the cortical spectrin network in untreated cells and the collapse of the α II/ β II-spectrin-based cytoskeleton into condensed foci in cells undergoing apoptosis (arrows). These changes are largely blocked by caspase inhibitor. Results shown are representative of four experiments. Scale bar = 10 μ m.

structural repeat unit 14,³ generating the immunoreactive C-terminal fragment α II-BDP120 (12). This cleavage also appears to liberate the α II-BDP35 fragment from α II-BDP150. No other caspase-induced cleavages in α II-spectrin were detected.

The proteolytic cascade of β II-spectrin is more complex. Unlike the case for calpain (19),² caspase 3 cleavage of β II-spectrin proceeds rapidly and in parallel with its action on α II-spectrin. The initial cleavage is at DEVD¹⁴⁵⁷*S¹⁴⁵⁸, generating β II-BDP110. This site is adjacent to the prominent calpain cleavage site in β II-spectrin.² Although inaccessible to sequencing and lacking immunoreactivity to pAb 10D, this cleavage also appears to liberate an intact \approx 165-kDa amino-terminal fragment (β II-BDP165, presumably residues 1–1457, calculated M_r of 170,061). This fragment is visible by Coomassie Blue staining (Fig. 4). The D¹⁴⁵⁷*S¹⁴⁵⁸ site appears to be an especially favorable one for cleavage, and indeed, the cognate DEVD sequence is identical to that in poly(ADP-ribose)polymerase, the classic caspase 3 substrate. Other cleavages appear, albeit more slowly. β II-BDP85 arises by a second C-terminal cleavage, probably at ETVD²¹⁴⁶*T (based on the cleavage pattern of the β II_{13-CA} fusion peptide) (Table I). The conservative substitution of Glu for Asp at the P4 position may account for the lower sensitivity of this site to caspase 3. Curiously, caspase 7 reportedly accepts a P4 Asp more readily than does caspase 3 (43), yet less β II-BDP85 (*versus* α II-BDP110) is generated by caspase 7 (Fig. 3), possibly highlighting the influence of complex conformational determinants in ultimately determining substrate specificity (44). Other β II-spectrin cleavage sites were also identified (Table I), and additional β II-spectrin cleavages may exist. However, our ability to detect such cleavages is

³ C. D. Cianci, Z. Zhang, and J. S. Morrow, submitted for publication.

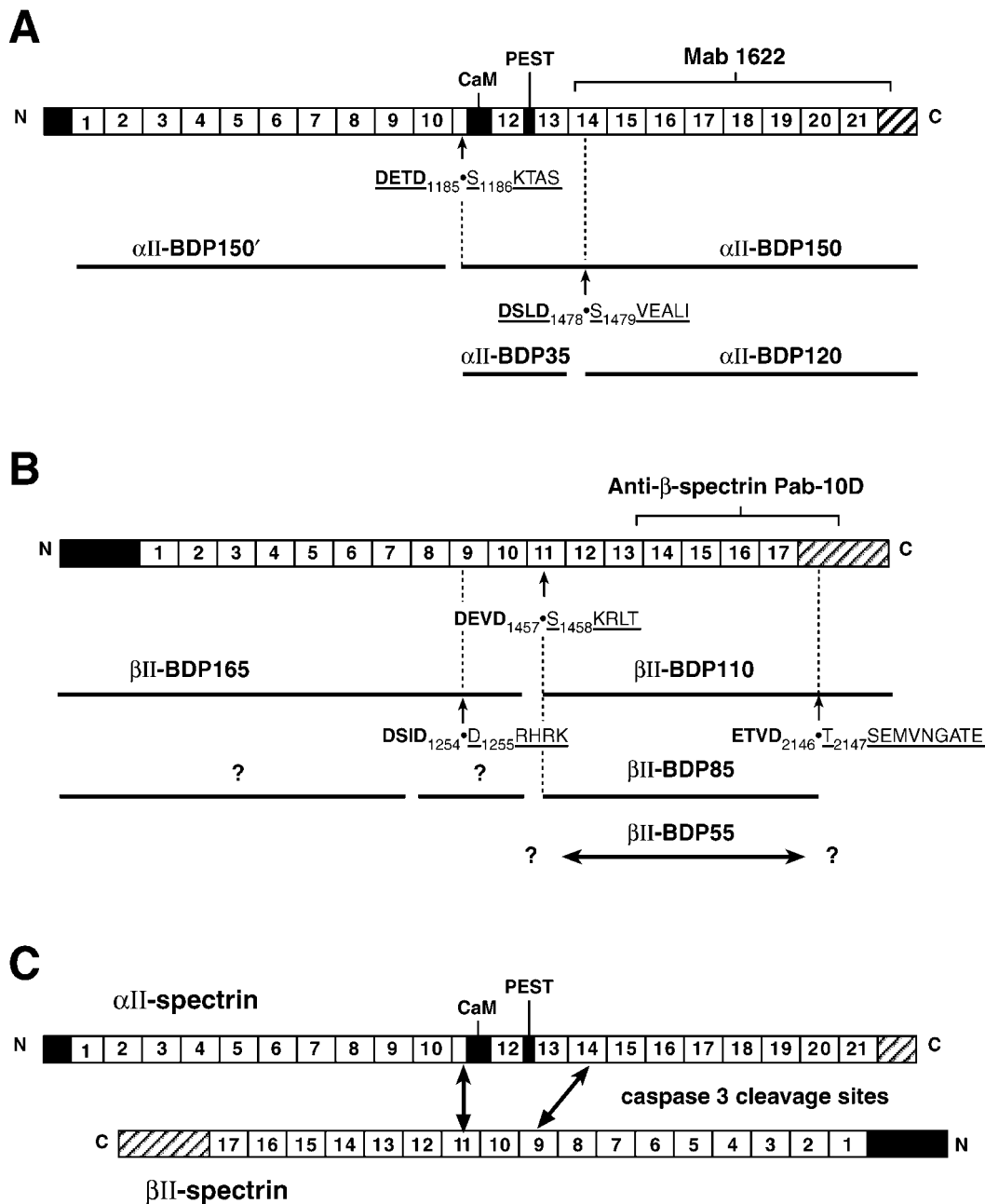


FIG. 8. Caspase digestion cascade for α II- and β II-spectrins. Depicted are the caspase 3 cleavages in α II-spectrin (A) and β II-spectrin (B) and their relative sequence of appearance based on the kinetics of digestion in SY5Y cells (see Fig. 1). Also depicted is the position of the cleavage fragments in relation to the tripartite domain structure of each spectrin subunit and the ≈ 106 -residue repeat structure characteristic of domain II (26). For definitively identified cleavages, the sequence flanking the cleavage site is given. Fragments without sequence verification but confirmed on the basis of their immunoreactivity are marked only by their apparent size in kDa. Other cleavages expected, but not identified, are marked with a *question mark*. Note that caspase cleavage site DETD*SK in α II-spectrin is located just proximal to the calmodulin (CaM)-binding domain in repeat 11. The PEST sequence (solid box) is located in repeat 12. The second caspase cleavage site in α II-spectrin is located in repeat 14 (DSLSD*SV). For β II-spectrin, the favored caspase cleavage is at DEVD*SK within repeat 11. This cleavage liberates an ≈ 165 -kDa fragment from the N-terminal portion of the molecule; further characterization of this fragment or subsequent cleavages has been hampered by the lack of immunoreactivity of pAb 10D with this portion of β II-spectrin. The second caspase cleavage site in β II-spectrin (ETVD*TS) is in the C-terminal region (hatched box). A minor cleavage (DSID*DR) was also identified in repeat 9 (dashed lines), based on studies with the GST fusion proteins. Also shown is a schematic illustration of the probable relationship of the major digestion sites in the $\alpha\beta$ -spectrin heterodimer (C). The structural depiction of the spectrin dimer and the alignment of the two subunits with respect to each other are adapted from Speicher *et al.* (14). Mab, monoclonal antibody.

limited by the lack of reactivity of pAb 10D for epitopes upstream of repeat 13 in β II-spectrin.

A central challenge in apoptosis research is understanding the mechanisms by which apoptotic cascades are initiated and effected. The results presented here offer potentially important insights into this process. Caspase inhibitors block not only the proteolytic actions of the enzyme, but also the cell death process itself (10, 11, 45). The action of caspase must therefore be

linked directly or indirectly to the phenotypic manifestation of apoptosis. Given the fundamental role of the cortical spectrin skeleton in maintaining membrane organization and integrity, we propose that the cleavage of spectrin, and particularly β II-spectrin, constitutes a critical, necessary, and sufficient step linking caspase activation to cell death. Several observations support this notion. (i) Only upon cleavage of β II-spectrin (*versus* α II-spectrin) are oligomeric spectrin-actin complexes disso-

ciated *in vitro* (20). (ii) Protease cleavage of β II-spectrin leads to loss of its ankyrin-independent membrane binding activity (46). (iii) β II-Spectrin harbors almost all recognized functional domains in the molecule (26). (iv) Mutations or modifications that disrupt the spectrin membrane skeleton universally lead to membrane disorder and disruption (26). (v) Gene knockouts of α II- or β II-spectrin are uniformly lethal (47, 48). (vi) The spectrin-actin skeleton links to several classes of intercellular adhesion molecules and may be required for their cell-cell adhesive activity (47, 49–52). (vii) Proteolytic cleavage of α II-spectrin alone, such as by calpain under conditions of physiologic stimulation, does not lead to cell death (15).² Thus, one can envision a process whereby limited cleavage of α II-spectrin alone, such as by calpain after *N*-methyl-D-aspartic acid receptor stimulation, is a physiologic process associated with skeletal plasticity, but is not *per se* lethal to cells. However, under pathologic conditions, either excessive stimulation of calpain (19)² or the activation of caspase degrades β II-spectrin, with consequential and rapid lethality. It is also worth noting that this hypothesis predicts that cells activated by limited proteolysis of α II-spectrin, such as by calpain, may be more susceptible to subsequent β II-spectrin degradation (and therefore apoptosis). This putative two-step process (α II-spectrin followed conditionally by β II-spectrin cleavage) would not be expected in cells directly triggered to apoptose (*e.g.* Jurkat T-cells challenged with anti-Fas antibody), under which conditions caspase is activated exclusively (23, 24). Conversely, when apoptosis only conditionally follows other processes, such as in neuronal remodeling or tumor necrosis factor α -mediated T-cell apoptosis, calpain may begin the process, to be followed conditionally by caspase (or sustained calpain activity) (22, 24).

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