THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 35, Issue of August 28, pp. 22490–22497, 1998
Printed in U.S.A.
© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.

Simultaneous Degradation of αII- and βII-Spectrin by Caspase 3 (CPP32) in Apoptotic Cells*

(Received for publication, December 2, 1997, and in revised form, May 19, 1998)

Kevin K. W. Wang‡§¶, Rand Posmantur‖, Rathna Nath¶, Kim McGinnis‡, Margaret Whitton, Robert V. Talanian‡‡, Susan B. Glantz§§, and Jon S. Morrow§§**

*From the ¶Laboratory of Neuro-biochemistry, Department of Neuroscience Therapeutics and the Departments of ‖Immunopathology and **Chemistry, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, Michigan 48105, the ‡Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan 48106, the ‡‡BASF Bioresearch Corporation, Worcester, Massachusetts 01605, and the §§Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510*

The degradation of αII- and βII-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 αII- and βII-subunits. Major C-terminal αII-spectrin BDPs were detected at ≈150, 145, and 120 kDa (αII-BDP150, αII-BDP145, and αII-BDP120, respectively); major C-terminal βII-spectrin BDPs were at ≈110 and 85 kDa (βII-BDP110 and βII-BDP85, respectively). N-terminal sequencing of the major fragments produced *in vitro* by caspase 3 revealed that αII-BDP150 and αII-BDP120 were generated by cleavages at DETD^{1185}*S^{1186} and DSLD^{1478}*S^{1479}, respectively. For βII-spectrin, a major caspase site was detected at DEVD^{1457}*S^{1458}, and both βII-BDP110 and βII-BDP85 shared a common N-terminal sequence starting with Ser^{1458}. An additional cleavage site near the C terminus, at ETVD^{2146}*S^{2147}, was found to account for βII-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 αII- and βII-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-1β-converting enzyme (ICE)[1]-like protease 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 αII-spectrin, the major component of the cortical membrane skeleton. In neurons, calcium-activated calpain cleavage of αII-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, αII-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 αII-spectrin bestows calmodulin regulation on oligomeric spectrin-actin complexes, but does not dissociate them (unless βII-spectrin is also cleaved) (19, 20). In addition to the action of calpain, αII-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 Tyr^{1176}–Gly^{1177} bond (VY*GMMPR) in αII-spectrin (27), calpain also targets several additional sites in both αII- and βII-spectrin (27).[2] In the present report, we demonstrate the specific sites of caspase 3 cleavage within both αII- and βII-spectrin and show that during apoptotic induction in neuroblastoma SH-SY5Y cells, it is caspase 3 that most rapidly cleaves not only αII-spectrin, but

* This work was supported in part by grants from the National Institutes of Health (to J. S. M.) and by a National Service Research Award fellowship (to S. B. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Lab. of Neuro-biochemistry, Department of Neuroscience Therapeutics, Parke-Davis Pharmaceutical Research, Div. of Warner-Lambert Co., 2800 Plymouth Rd., Ann Arbor, MI 48105. Fax: 734-622-7178; E-mail: Kevin.Wang@WL.com.

[1] The abbreviations used are: ICE, interleukin-1β-converting enzyme; MTX, maitotoxin; Z-D-DCB, benzyloxycarbonyl-Asp-$CH_2OC(O)$-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; αII-BDP, αII-spectrin breakdown product; βII-BDP, βII-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.

This paper is available on line at http://www.jbc.org

also βII-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 (~2 × 10^6 cells/well) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/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 μM 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 spectrin[2] 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-αII-spectrin (pAb RAF-A) and anti-βII-spectrin (pAb 10D) antibodies, respectively.[2] Similar experiments were also carried out on GST fusion peptides representing various regions of αII- and βII-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-μl samples) (39). Alternatively, 40 μg/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 Cytoflor 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 μg) 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 μm) using a Tris/glycine buffer system in a semidry electrotransfer unit (Bio-Rad) at 20 mA for 1.5–2 h. αII-Spectrin was detected with monoclonal antibody 1622 (Chemicon International, Inc.), pAb RAF-A, or pAb RAF-B (34). βII-Spectrin was detected by pAb 10D, raised against a recombinant βII-spectrin peptide representing repeat unit 13 to residue 2204 within domain III ($\beta II_{13\text{-}C\Delta}$) (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 employed 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 Na_2HPO_4, and 14 mM KH_2PO_4 (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 $NaPO_4$ (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 αII- and βII-Spectrin Are Degraded in Apoptotic SY5Y Cells—Our previous studies have established that SY5Y cells subjected to staurosporine (0.5 μM) 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 αII- and βII-spectrin in staurosporine-treated cells was detected by 1 h and continued progressively for 24 h (Fig. 1*A*). This loss was mirrored by increased levels of αII-spectrin BDPs at ≈150, 145, and 120 kDa (αII-BDP150, αII-BDP145, and αII-BDP120, respectively). βII-Spectrin was also simultaneously degraded into two major immunoreactive fragments at ≈110 and 85 kDa (βII-BDP110 and βII-BDP85, respectively). A minor βII-BDP was also evident at ≈55 kDa (βII-BDP55) (Fig. 1*B*).

The time course of the breakdown of both αII- and βII-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).

$$A \xrightarrow{k_A} B \xrightarrow{k_B} C \qquad \text{(Eq. 1)}$$

Scaling data to unit maximum value for *A* (the starting value of αII- or βII-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).

$$B = \frac{k_A(e^{-k_At} - e^{-k_Bt})}{(k_B - k_A)} \qquad \text{(Eq. 2)}$$

$$C = (1 - e^{-k_At}) - \frac{k_A(e^{-k_At} - e^{-k_Bt})}{(k_B - k_A)} \qquad \text{(Eq. 3)}$$

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

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

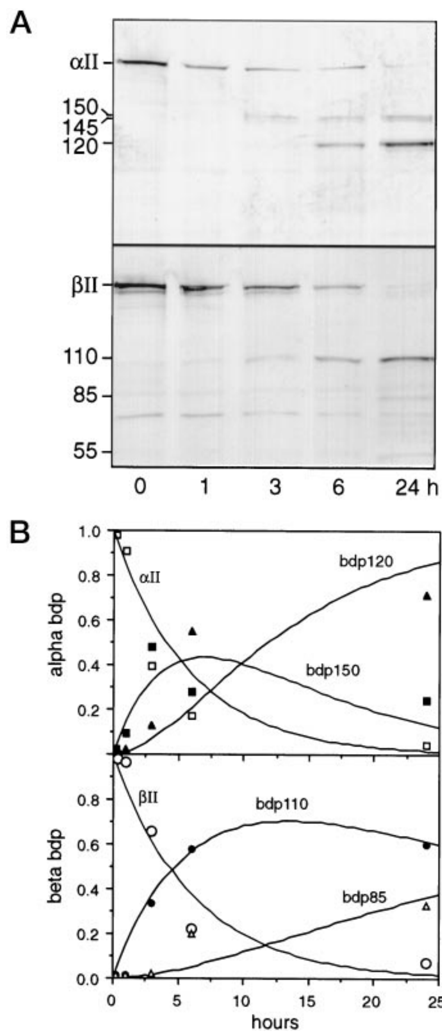

FIG. 1. **Time course of αII- and βII-spectrin breakdown in apoptotic SY5Y cells.** *A,* SH-SY5Y cells were challenged with 0.5 μM staurosporine for 0–24 h. Total cellular protein was extracted and analyzed (15 μg/lane) by SDS-PAGE and Western blotting using monoclonal antibody 1622 (anti-αII-spectrin) or pAb 10D (anti-βII-spectrin). The position of intact αII-spectrin (*upper panel*) or βII-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 αII-spectrin (*open squares*), αII-BDP150 (*closed squares*), αII-BDP120 (*closed triangles*), βII-spectrin (*open circles*), βII-BDP110 (*closed circles*), and βII-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 αII-spectrin and 0.1269 for βII-spectrin.

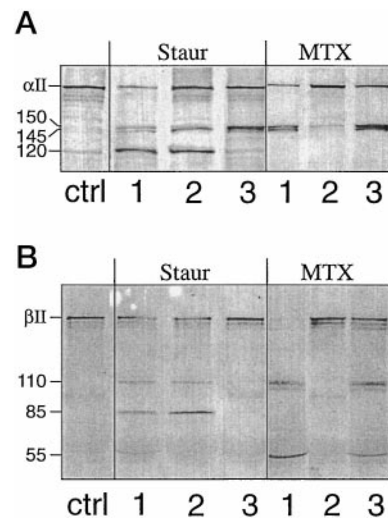

FIG. 2. **Staurosporine-induced apoptosis in SY5Y cells is mainly caspase-mediated.** Shown are the effects of caspase and calpain inhibitors on αII-spectrin (*A*) and βII-spectrin (*B*) breakdown in SY5Y cells. Cells were either untreated (*ctrl*) or challenged with 0.5 μM staurosporine (*Staur*) for 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 μM calpain inhibitor I (*lane 2*), or 50 μM 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.

hypersensitive region within αII-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 αII-BDP145, but did not affect the generation of the major product, αII-BDP120 (Fig. 2*A*). Conversely, Z-D-DCB, a caspase inhibitor, blocked the appearance of αII-BDP120. Both inhibitors slowed the overall generation of αII-BDPs, and thus, it appeared that both proteases contributed in some measure to the generation of αII-spectrin BDPs. With respect to βII-spectrin, calpain inhibitor I had little effect on βII-spectrin degradation compared with Z-D-DCB, which blocked the cleavage of βII-spectrin almost completely (Fig. 2*B*). 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 αII- and βII-spectrin coupled with the generation of αII-BDP150, βII-BDP110, and βII-BDP55. On the other hand, the αII-BDP120 and βII-BDP85 products, characteristic of caspase 3 activity, were not formed. Z-D-DCB provided minimal protection, whereas calpain inhibitor I almost completely blocked both αII- and βII-spectrin breakdown in MTX-treated cells (Fig. 2). A βII-spectrin cleavage product of ≈110 kDa observed with MTX is similar in size to the caspase-generated βII-BDP110; based on the lack of βII-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 βII-spectrin as part of the cell death event.

αII/βII-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 αII-BDP150, presum-

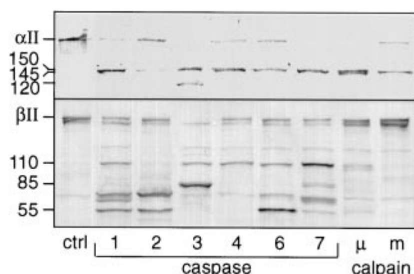

FIG. 3. **Different caspases yield distinct spectrin cleavage patterns.** SY5Y cellular extracts (20 μg of protein) were subjected to *in vitro* digestion with 0. 5 μg each of caspases 1–4 and 6–8 for 2 h. Alternatively, the same extracts were digested with 0.5 μg of μ-calpain or m-calpain for 30 min 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 αII-spectrin (*upper panel*) and βII-spectrin (*lower panel*) are as indicated (in kDa). Results shown are representative of three experiments.

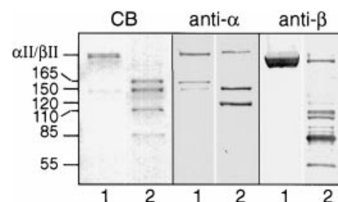

FIG. 4. ***In vitro* digestion of αII/βII-spectrin by caspase 3.** Bovine αII/βII-spectrin heterotetramer (20 μg) was digested with 1 μg of caspase 3 (CPP32, beta form) for 1 h at 25 °C. The samples were analyzed by PAGE followed by Coomassie Blue staining (*CB*) or probed with antibody against αII-spectrin (*anti-α*) or βII-spectrin (*anti-β*). Undigested material is in *lane 1,* and digested material is in *lane 2*. The intact αII- and βII-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 ≈165-kDa band, which arises from the N-terminal half of βII-spectrin. pAb 10D does not react with this portion of βII-spectrin.

ably due to cleavage within αII-spectrin's hypersensitive site. In contrast, only caspase 3 produced significant levels of αII-BDP120. All caspases also readily digested βII-spectrin, but differed significantly in the ratio of the major βII-spectrin fragments generated (Fig. 3). For example, the dominant products generated by caspases 2, 3, 6, and 7 were βII-BDP70, βII-BDP85, βII-BDP55, and βII-BDP110, respectively. Again, only caspase 3 produced significant levels of both βII-BDP110 and βII-BDP85. While additional βII-spectrin BDPs no doubt existed in these experiments that were not visualized by pAb 10D (which is directed to the C-terminal third of βII-spectrin), these findings establish that caspases display characteristic differences in their relative specificity and activity *vis-á-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 αII-spectrin often showed the greatest activity against βII-spectrin. Interestingly, αII-BDP120, βII-BDP110, and βII-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 αII- and βII-Spectrin at Multiple Consensus Recognition Sites—The sites of calpain cleavage in both αII- and βII-spectrin have been identified (27).[2] To identify the precise sites at which caspase 3 cleaves spectrin, purified bovine brain αII/βII-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 ≈165 to ≈85 kDa on Coomassie Blue-stained gels (Fig. 4). Two of these products (αII-BDP150 and αII-BDP120) reacted with monoclonal antibody 1622, indicating their origin from the αII-subunit. Conversely, pAb 10D detected βII-fragments at ≈110, ≈100, ≈85, and ≈55 kDa. Only the Coomassie Blue-stained fragment at ≈165 kDa was unaccounted for on the Western blots; presumably this represents a βII-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 αII-BDP145 generated by calpain).

N-terminal sequencing identified the origin of several major caspase 3-generated fragments. Due to the presence of multiple 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 αII-BDP150 fragment mapped to the sequence DETD^{1185}*S^{1186}KTASP 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 (VY^{1176}*G^{1177}MMP) and immediately proximal to the calmodulin-binding domain (residues 1187–1206) (27). It is also likely that the N-terminal half of αII-spectrin was present within the αII-BDP150 band, based on analogy with the cleavage of αII-spectrin by calpain (7, 36).[2] However, given the blocked N terminus of αII-spectrin, this fragment did not appear in the microsequencing results. The αII-BDP120 fragment mapped to a second caspase 3 cleavage site (DSLD^{1478}*S^{1479}EALIKKHE) in repeat 14 of αII-spectrin. The fragment liberated from αII-BDP150 to yield αII-BDP120 appeared in αII-BDP35 (Table I). Both the βII-BDP110 and βII-BDP85 fragments shared a common N-terminal sequence, placing this site of cleavage in repeat 11 of βII-spectrin (DEVD^{1457}*S^{1458}KRLTVQT). Reliable sequence information was not obtained from βII-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 αII-spectrin (from repeat 9 through the C terminus) were digested with caspase 3 (Fig. 5). The GST-αII_{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. 5*A*, *arrows*). The anti-GST antibodies reacted with the 75-kDa fragment, indicating its origin from the N-terminal half of the fusion peptide (Fig. 5*B*). End sequencing of the 20-kDa fragment yielded -SKTASPWK-SAR, identical to the cleavage site generating αII-BDP150 from caspase treatment of the intact heterotetramer (Table I). Similarly, digestion of GST-αII_{13-18} generated two fragments of ≈50 and ≈45 kDa (Fig. 5*A*). Antibodies against GST and αII-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 αII-BDP120 cleavage site (Table I). Finally, the GST-$\alpha II_{18\text{-}C}$ polypeptide resisted digestion with caspase 3.

Two GST fusion polypeptides representing regions of βII-spectrin were also subjected to caspase 3 digestion (Fig. 6). GST-βII_{8-13} extended from repeat 8 through repeat 13, and GST-$\beta II_{13\text{-}C\Delta}$ 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 αIIΣ1-spectrin (GenBank™ U83867) (footnote 3) and βII-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				
αII-BDP150′ [a]	(MDPSGVKVLE)	(Start of αII-spectrin, assumed)	Asp^{1185}	136,833
αII-BDP150	-SKTAXPWKXA	DETD^{1185}*S^{1186}KTASPWKSA	Asn^{2477}	148,278
αII-BDP120	-SVEALIKKH	DSLD^{1478}*S^{1479}VEALIKKHED	Asn^{2477}	114,602
αII-BDP35	-SKTASPWKSAR	DETD^{1185}*S^{1186}KTASPWKSAR	Asp^{1478}	33,693
βII-BDP165	(MTTTVATD)	(Start of βII-spectrin, assumed)	Asp^{1457}	170,061
βII-BDP110	-SKRLTVQTXF	DEVD^{1457}*S^{1458}KRLTVQTKFM	Lys^{2364}	104,614
βII-BDP85	-SKRLTVQTKF	DEVD^{1457}*S^{1458}KRLTVQTKF	Asp^{2146}	80,404
βII-BDP55	ND[b]			
GST constructs				
GST-αII$_{9–12}$		(Start of αII-sequence at Glu^{837})	Ser^{1267}	76,331
75 kDa	-MSPILGYWKI	N terminus of GST	Asp^{1185}	66,818
20 kDa	-SKTASPWXSA	DETD^{1185}*S^{1186}KTASPWKSAR	Ser^{1267}	9,536
GST-αII$_{13–18}$		(Start of αII-sequence at Ala^{1333})	Lys^{1928}	95,113
50 kDa	-SVEALIKKH	DSLD^{1478}*S^{1479}VEALIKKHED	Lys^{1928}	51,292
45 kDa	-MSPILGYWKI	N terminus of GST	Asp^{1478}	43,838
GST-αII$_{18–C}$	(No BDP detected)	(Start of αII-sequence at position 1929)	Asn^{2477}	90,291
GST-βII$_{8–13}$		(Start of βII-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-βII$_{13–CΔ}$		(Start of βII-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 αII-BDP150 band, representing αII-spectrin residues 1–1185; this product (αII-BDP150′) cannot be detected by end sequencing since the N terminus of spectrin is methylated.
[b] ND, not determined.

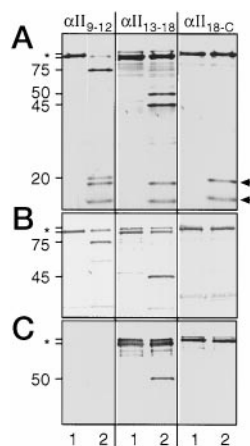

FIG. 5. **Digestion of GST-αII-spectrin fusion proteins by caspase 3.** GST fusion proteins αII$_{9–12}$, αII$_{13–18}$, and αII$_{18\text{-C}}$ (15 μg each) were either left untreated (*lane 1*) or digested with 0.8 μg of caspase 3 for 1 h at 25 °C (*lane 2*). The samples were analyzed by SDS-PAGE followed by staining with Coomassie Blue (*A*) or probed with antibodies against either GST (*B*) or αII-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-βII$_{8–13}$ (≈72 kDa) was rapidly cleaved to an ≈48-kDa fragment and several bands around 22–20 kDa (Fig. 6*A*). The 48- and 21-kDa bands were immunoreactive with anti-GST antibody and contained native GST N-terminal sequence (Fig. 6*B*). The N termini of the 22-kDa fragment and the 20-kDa minor fragment were generated by the cleavages DEVD^{1457}*S^{1458}KRLTVQTKF and DSID^{1254}*D^{1255}RHRKNRETA, respectively (Table I). Evaluation of GST-βII$_{13\text{-C}Δ}$ (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 ≈77 and ≈8 kDa were identified as caspase-specific (Fig. 6*C*). 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 ETVD^{2146}*T^{2147}SEMVNGATE (Table I).

Immunofluorescence Staining—The integrity of the spectrin-based cytoskeleton in apoptotic SY5Y cells was evaluated by indirect immunofluorescence using αII-spectrin-specific (pAb RAF-A) and βII-spectrin-specific (pAb 10D) antibodies. Control cells demonstrated a subplasmalemma distribution of αII/βII-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 αII- and βII-spectrin breakdown occurred (Fig. 1), and αII-spectrin collapsed into localized cytosolic aggregates coincident with cell shrinkage and fragmentation of the cell processes (Fig. 7*E*). Cytosolic spectrin was also prominent in dissociated apoptotic bodies. The changes in βII-spectrin paralleled those in αII-spectrin (Fig. 7*H*). These changes were blocked partially by Z-D-DCB cotreatment (Fig. 7, *F* and *I*).

DISCUSSION

Beginning with the observation that αII-spectrin cleavage is tightly coupled to the process of apoptosis in lymphocytes (21), several studies have noted the generation of an ≈150-kDa BDP of αII-spectrin (12, 22–25). This is perhaps not a surprising observation given the extreme sensitivity of αII-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 βII-spectrin is cleaved as rapidly as αII-spectrin during staurosporine-induced apoptosis; (ii) that the primary protease acting on spectrin under these conditions is

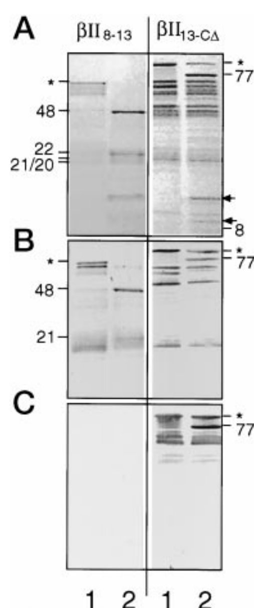

FIG. 6. **Digestion of GST-βII-spectrin fusion proteins by caspase 3.** GST fusion proteins βII_{8-13} and $\beta II_{13\text{-}C\Delta}$ (15 μg each) were either left untreated (*lane 1*) or digested with 0.8 μg of caspase 3 for 1 h at 25 °C (*lane 2*). The samples were analyzed by SDS-PAGE followed by staining with Coomassie Blue (*A*) or probed with antibody against either GST (*B*) or βII-spectrin (*C*). As expected, there was no immunoreactivity of the βII_{8-13} peptide or its fragments with the anti-βII-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.

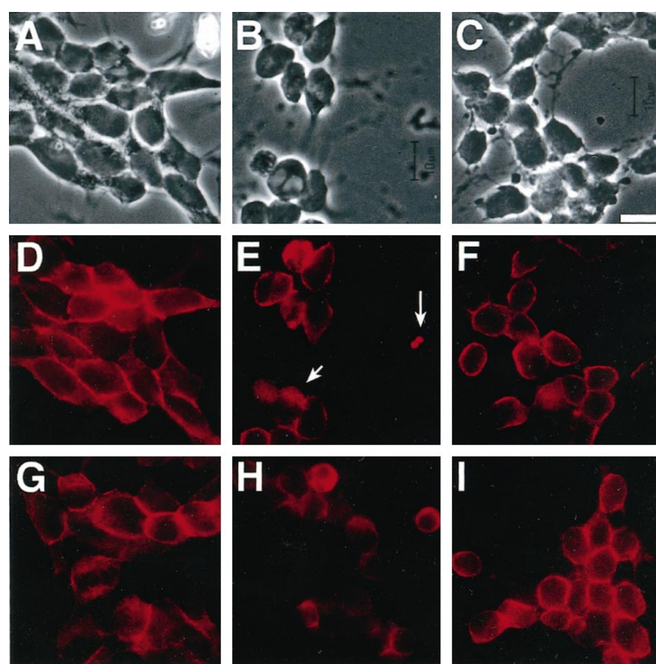

FIG. 7. **Immunofluorescence of αII- and βII-spectrins in apoptotic SH-SY5Y cells.** SY5Y cells were either untreated (*A*, *D*, and *G*) or challenged with 0.5 μM staurosporine for 5 h in the absence (*B*, *E*, and *H*) or presence (*C*, *F*, and *I*) of 100 μM Z-D-DCB. The cells were then fixed and examined by phase microscopy (*A–C*) or subjected to immunofluorescence analysis with antibodies to αII-spectrin (pAb RAF-A; *D–F*) and βII-spectrin (pAb 10D; *G–I*). Note the uniform labeling of the cortical spectrin network in untreated cells and the collapse of the αII/βII-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 μm.

caspase 3; (iii) that the cleavage of βII-spectrin correlates temporally with the onset of apoptosis; and (iv) that caspase 3 cleaves at precise and characteristic sequences within both αII- and βII-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 βII-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 αII- and βII-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 D*XX*D*S is strongly preferred as an endogenous substrate for caspase 3. However, the heterodimeric unit of spectrin contains 36 D*XX*D sequence motifs, yet caspase 3 efficiently cleaves αII-spectrin at only two sites and βII-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 αII-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 αII-spectrin occurs between Asp^{1185} and Ser^{1186}. 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 αII-spectrin approximately in half. Subsequently, caspase cuts αII-spectrin between D^{1478}*S^{1479}, a site positioned between helices A and B in structural repeat unit 14,[3] generating the immunoreactive C-terminal fragment αII -BDP120 (12). This cleavage also appears to liberate the αII-BDP35 fragment from αII-BDP150. No other caspase-induced cleavages in αII-spectrin were detected.

The proteolytic cascade of βII-spectrin is more complex. Unlike the case for calpain (19),[2] caspase 3 cleavage of βII-spectrin proceeds rapidly and in parallel with its action on αII-spectrin. The initial cleavage is at $DEVD^{1457}$*S^{1458}, generating βII-BDP110. This site is adjacent to the prominent calpain cleavage site in βII-spectrin.[2] Although inaccessible to sequencing and lacking immunoreactivity to pAb 10D, this cleavage also appears to liberate an intact ≈165-kDa amino-terminal fragment (βII-BDP165, presumably residues 1–1457, calculated M_r of 170,061). This fragment is visible by Coomassie Blue staining (Fig. 4). The D^{1457}*S^{1458} 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. βII-BDP85 arises by a second C-terminal cleavage, probably at $ETVD^{2146}$*T (based on the cleavage pattern of the $\beta II_{13\text{-}C\Delta}$ 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 βII-BDP85 (*versus* αII-BDP110) is generated by caspase 7 (Fig. 3), possibly highlighting the influence of complex conformational determinants in ultimately determining substrate specificity (44). Other βII-spectrin cleavage sites were also identified (Table I), and additional βII-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.

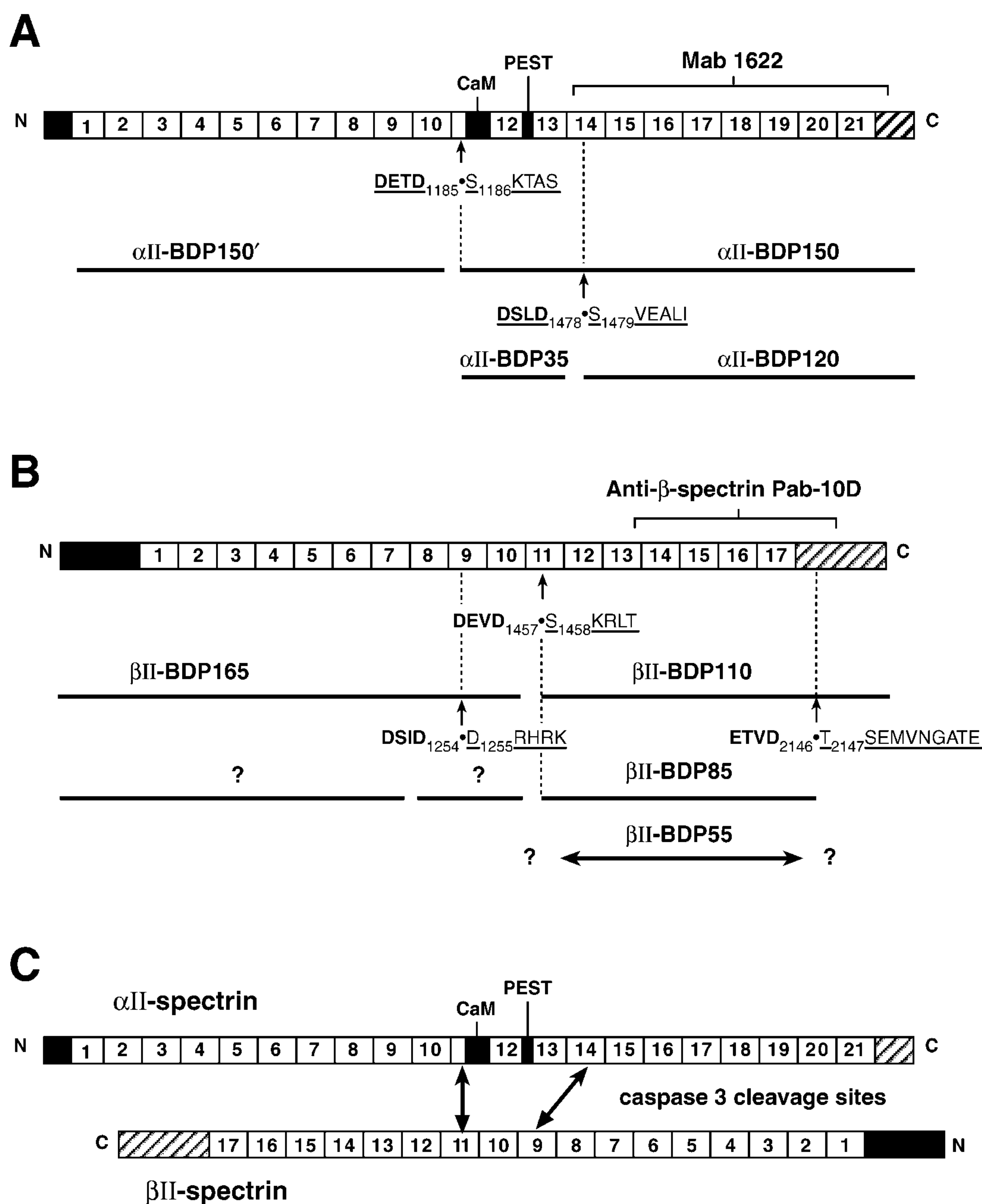

FIG. 8. **Caspase digestion cascade for αII- and βII-spectrins.** Depicted are the caspase 3 cleavages in αII-spectrin (*A*) and βII-spectrin (*B*) and their relative sequence of appearance based on the kinetics of digestion in SY5Y cells (see Fig. 1). Also depicted is the position of the cleavage fragments 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 αII-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 αII-spectrin is located in repeat 14 (DSLD*SV). For βII-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 βII-spectrin. The second caspase cleavage site in βII-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 αβ-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 βII-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 βII-spectrin, constitutes a critical, necessary, and sufficient step linking caspase activation to cell death. Several observations support this notion. (i) Only upon cleavage of βII-spectrin (*versus* αII-spectrin) are oligomeric spectrin-actin complexes disso-

ciated *in vitro* (20). (ii) Protease cleavage of βII-spectrin leads to loss of its ankyrin-independent membrane binding activity (46). (iii) βII-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 αII- or βII-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 αII-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 αII-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 βII-spectrin, with consequential and rapid lethality. It is also worth noting that this hypothesis predicts that cells activated by limited proteolysis of αII-spectrin, such as by calpain, may be more susceptible to subsequent βII-spectrin degradation (and therefore apoptosis). This putative two-step process (αII-spectrin followed conditionally by βII-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).

Acknowledgments—We are in debt to John Gilmore, Bradley Caprathe, and Dr. Sheryl Hays (Department of Chemistry, Parke-Davis Pharmaceutical Research) for synthesizing Z-D-DCB and to Dr. Hamish Allen (BASF Bioresearch Corp.) for helpful discussion. We thank Drs. Deepti Pradan and Carol Cianci and Paul Stabach for assistance with the recombinant spectrin constructs and protein purification.

REFERENCES

1. Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996) *Cell* **87,** 171
2. Wang, L., Miura, M., Bergeron, L., Zhu, H., and Yuan, J. (1994) *Cell* **78,** 739–750
3. Fraser, A., and Evan, G. (1996) *Cell* **85,** 781–784
4. Vaux, D. L., and Strasser, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 2239–2244
5. 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) *Nature* **376,** 37–43
6. Kuida, K., Zheng, T. S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P., and Flavell, R. A. (1996) *Nature* **384,** 368–372
7. Glantz, S. B., and Morrow, J. S. (1996) in *Tissue Oxygen Deprivation: Developmental, Molecular and Integrated Function* (Haddad, G. G., and Lister, G., eds) Vol. 95, pp. 153–192, Marcel Dekker, Inc., New York
8. Murachi, T. (1989) *Biochem. Int.* **18,** 263–294
9. Squier, M. K., Miller, A. C., Malkinson, A. M., and Cohen, J. J. (1994) *J. Cell. Physiol.* **159,** 229–237
10. Sarin, A., Adams, D. H., and Henkart, P. A. (1993) *J. Exp. Med.* **178,** 1693–1700
11. Sarin, A., Clerici, M., Blatt, S. P., Hendrix, C. W., Shearer, G. M., and Henkart, P. A. (1994) *J. Immunol.* **153,** 862–872
12. Nath, R., Raser, K. J., Stafford, D., Hajimohammadreza, I., Posner, A., Allen, H., Talanian, R. V., Yuen, P., Gilbertsen, R. B., and Wang, K. K. W. (1996) *Biochem. J.* **319,** 683–690
13. del Cerro, S., Arai, A., Kessler, M., Bahr, B. A., Vanderklish, P., Rivera, S., and Lynch, G. (1994) *Neurosci. Lett.* **167,** 149–152
14. Speicher, D. W., DeSilva, T. M., Speicher, K. D., Ursitti, J. A., Hembach, P., and Weglarz, L. (1993) *J. Biol. Chem.* **268,** 4227–4235
15. Di Stasi, A. M., Gallo, V., Ceccarini, M., and Petrucci, T. C. (1991) *Neuron* **6,** 445–454
16. Lynch, G., Kessler, M., Arai, A., and Larson, J. (1990) *Prog. Brain Res.* **83,** 233–250
17. Lynch, G., and Baudry, M. (1987) *Brain Res. Bull.* **18,** 809–815
18. Siman, R., Noszek, J. C., and Kegerise, C. (1989) *J. Neurosci.* **9,** 1579–1590
19. Harris, A. S., Croall, D. E., and Morrow, J. S. (1989) *J. Biol. Chem.* **264,** 17401–17408
20. Harris, A. S., and Morrow, J. S. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3009–3013
21. Martin, S. J., O'Brien, G. A., Nishioka, W. K., McGahon, A. J., Mahboubi, A., Saido, T. C., and Green, D. R. (1995) *J. Biol. Chem.* **270,** 6425–6428
22. Nath, R., Raser, K. J., McGinnis, K., Nadimpalli, R., Stafford, D., and Wang, K. K. (1996) *Neuroreport* **8,** 249–255
23. Greidinger, E. L., Miller, D. K., Yamin, T. T., Casciola-Rosen, L., and Rosen, A. (1996) *FEBS Lett.* **390,** 299–303
24. Vanags, D. M., Porn-Ares, M. I., Coppola, S., Burgess, D. H., and Orrenius, S. (1996) *J. Biol. Chem.* **271,** 31075–31085
25. Cryns, V. L., Bergeron, L., Zhu, H., Li, H., and Yuan, J. (1996) *J. Biol. Chem.* **271,** 31277–31282
26. Morrow, J. S., Rimm, D. L., Kennedy, S. P., Cianci, C. D., Sinard, J. H., and Weed, S. A. (1997) in *Handbook of Physiology* (Hoffman, J., and Jamieson, J., eds) Vol. 11, pp. 485–540, Oxford University Press, London
27. Harris, A. S., Croall, D., and Morrow, J. S. (1988) *J. Biol. Chem.* **263,** 15754–15761
28. Wang, K. K. W., Nath, R., Raser, K. J., and Hajimohammadreza, I. (1996) *Arch. Biochem. Biophys.* **331,** 208–214
29. Bertrand, R., Solary, E., O'Connor, P., Kohn, K. W., and Pommier, Y. (1994) *Exp. Cell Res.* **211,** 314–321
30. 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) *J. Biol. Chem.* **270,** 15250–15256
31. Bump, N. J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferenz, C., Franklin, S., Ghayur, T., Li, P., Licari, P., Mankovich, J., Shi, L., Greenberg, A. H., Miller, L. K., and Wong, W. W. (1995) *Science* **269,** 1885–1888
32. Lombardo, C. R., Weed, S. A., Kennedy, S. P., Forget, B. G., and Morrow, J. S. (1994) *J. Biol. Chem.* **269,** 29212–29219
33. Wang, K. K., Posner, A., and Hajimohammadreza, I. (1996) *BioTechniques* **20,** 662–668
34. Harris, A. S., Anderson, J. P., Yurchenco, P. D., Green, L. A. D., Ainger, K. J., and Morrow, J. S. (1986) *J. Cell. Biochem.* **30,** 51–70
35. Posmantur, R., McGinnis, K., Nadimpalli, R., Gilbertsen, R. B., and Wang, K. K. W. (1997) *J. Neurochem.* **68,** 2328–2337
36. Harris, A. S., and Morrow, J. S. (1988) *J. Neurosci.* **8,** 2640–2651
37. Faleiro, L., Kobayashi, R., Fearnhead, H., and Lazebnik, Y. (1997) *EMBO J.* **16,** 2271–2281
38. Zhivotovsky, B., Burgess, D. H., Vanags, D. M., and Orrenius, S. (1997) *Biochem. Biophys. Res. Commun.* **230,** 481–488
39. Wang, X., Zelenski, N. G., Yang, J., Sakai, J., Brown, M. S., and Goldstein, J. L. (1996) *EMBO J.* **15,** 1012–1020
40. Na, S., Chuang, T. H., Cunningham, A., Turi, T. G., Hanke, J. H., Bokoch, G. M., and Danley, D. E. (1996) *J. Biol. Chem.* **271,** 11209–11213
41. Goldberg, Y. P., Nicholson, D. W., Rasper, D. M., Kalchman, M. A., Koide, H. B., Graham, R. K., Bromm, M., Kazemi-Esfarjani, P., Thornberry, N. A., Vaillancourt, J. P., and Hayden, M. R. (1996) *Nat. Genet.* **13,** 442–449
42. Smith, D. B., Davern, K. M., Board, P. G., Tiu, W. U., Garcia, E. G., and Mitchell, G. F. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8703–8707
43. Talanian, R. V., Quinlan, C., Trautz, S., Hackett, M. C., Mankovich, J. A., Banach, D., Ghayur, T., Brady, K. D., and Wong, W. W. (1997) *J. Biol. Chem.* **272,** 9677–9682
44. Stabach, P. R., Cianci, C. D., Glantz, S. B., Zhang, Z., and Morrow, J. S. (1997) *Biochemistry* **36,** 57–65
45. Armstrong, R. C., Aja, T., Xiang, J., Gaur, S., Krebs, J. F., Hoang, K., Bai, X., Korsmeyer, S. J., Karanewsky, D. S., Fritz, L. C., and Tomaselli, K. J. (1996) *J. Biol. Chem.* **271,** 16850–16855
46. Hu, R.-J., and Bennett, V. (1991) *J. Biol. Chem.* **266,** 18200–18205
47. Lee, J. K., Coyne, R. S., Dubreuil, R. R., Goldstein, L. S., and Branton, D. (1993) *J. Cell Biol.* **123,** 1797–1809
48. Deng, H., Lee, J. K., Goldstein, L. S., and Branton, D. (1995) *J. Cell Biol.* **128,** 71–79
49. Lokeshwar, V. B., Fregien, N., and Bourguignon, L. Y. (1994) *J. Cell Biol.* **126,** 1099–1109
50. Nelson, W. J., Shore, E. M., Wang, A. Z., and Hammerton, R. W. (1990) *J. Cell Biol.* **110,** 349–357
51. Rimm, D. L., Koslov, E. R., Kebriaei, P., Cianci, C. D., and Morrow, J. S. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8813–8817
52. Devarajan, P., and Morrow, J. S. (1996) in *Membrane Protein-Cytoskeleton Complexes: Protein Interactions, Distributions and Functions* (Nelson, W. J., ed) Vol. 43, pp. 97–128, Academic Press, New York
53. Hu, R.-J., Watanabe, M., and Bennett, V. (1992) *J. Biol. Chem.* **267,** 18715–18722