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Transforming Growth Factor β Induces Caspase 3-independent Cleavage of αII-Spectrin (α-Fodrin) Coincident with Apoptosis*

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Transforming growth factor β (TGF-β) is a potent growth inhibitor and inducer of cell death in B-lymphocytes and is essential for immune regulation and maintenance of self-tolerance. In this report the mouse immature B cell line, WEHI 231, was used to examine the mechanisms involved in TGF-β-mediated apoptosis. Induction of apoptosis is detected as early as 8 h after TGF-β administration. Coincident with the onset of apoptosis, the cytoskeletal actin-binding protein, αII-spectrin (α-fodrin) is cleaved into 150-, 115-, and 110-kDa fragments. The broad spectrum caspase inhibitor (Boc-D-fmk (BD-fmk)) completely abolished TGF-β-induced apoptosis and αII-spectrin cleavage. Caspase 3, although present in WEHI 231 cells, was not activated by TGF-β, nor was its substrate, poly(ADP-ribose) polymerase. These results identify αII-spectrin as a novel substrate that is cleaved during TGF-β-induced apoptosis. Our data provide the first evidence of calpain and caspase 3-independent cleavage of αII-spectrin during apoptosis and suggests that TGF-β induces apoptosis and αII-spectrin cleavage via a potentially novel caspase. This report also provides the first direct evidence of caspase 3 activation in WEHI 231 cells and indicates that at least two distinct apoptotic pathways exist.

Elimination of immature B cells during development occurs via apoptosis and is essential for the induction and maintenance of immune tolerance (3). Failure to eliminate immature B cells results in lymphoproliferation. Strict regulation of lymphocyte maturation is critical in preventing cancer as well as autoimmune disease. Although establishing B cell tolerance is a critical process required to prevent autoimmune disease, the mechanisms and factors regulating this process are not well understood. The mouse immature B cell line, WEHI 231, has been used as an in vitro model of B cell tolerance (3–6) and apoptosis (5–9), and the growth regulator, transforming growth factor β (TGF-β),1 has been shown to induce apoptosis in these cells (5, 6, 10).

TGF-β is an important regulator of cell growth (13–18) and essential for the maintenance of normal immunological homeostasis (14, 17) and lymphocyte proliferation (9–12). Human and mouse lymphocytes (7–9), as well as many other cell types, respond to TGF-β by undergoing apoptosis (18–28); however, the mechanisms regulating this process are not well understood and appear to be cell-specific. The ability of TGF-β to induce apoptosis in immature B cells may be a major mechanism controlling lymphocyte growth and subsequently regulating the immune response. TGF-β-deficient mice exhibit extensive lymphocytic hyperproliferation and systemic lupus erythematosus-like autoantibodies (29, 30). The phenotypic consequences of TGF-β1 ablation in mice are similar to Sjögren’s syndrome, a human organ-specific autoimmune disease (29, 31–33). Patients with Sjögren’s syndrome develop with higher frequency malignant B cell lymphoma, and an even higher percentage develop pseudolymphoma (34). Recently, αII-spectrin(1, 2)2 has been identified as a candidate autoantigen believed to be a primary initiator of Sjögren’s syndrome (31).

αII-spectrin is a ubiquitous, heterodimeric actin-binding cytoskeletal protein postulated to play a vital role in the structural integrity and organization of cells. Plasma membrane blebbing, an early apoptotic phenotype, has been proposed to result as a consequence of the loss or cleavage of intact αII-spectrin (35). A cleavage product of αII-spectrin has been shown to induce characteristics similar to Sjögren’s syndrome in mice (34), although it is currently unclear how this cleavage product is formed. αII-spectrin has been shown to be cleaved into 150-, 145-, and 120-kDa fragments during apoptosis by calpain and/or caspase 3 and caspase 3 in human T cells, neurons, and hematopoietic cells (22, 35–38). The 150-kDa fragment arises by cleavage at a hypersensitive site present within the protein and is generated by different caspasess (22, 36). The 145-kDa fragment is generated in some but not all systems via activation of calpain, a calcium-dependent cysteine protease (37, 39). Generation of a 120-kDa fragment is specific for caspase cleavage (22, 35, 37, 38). In addition to calpain, the only reported proteases capable of cleaving αII-spectrin in vivo are caspase 3 and poly(ADP-ribose) polymerase (PARP)-sensitive, caspase 3-like proteases.

In the present study, we have investigated the pathways that mediate TGF-β-induced apoptosis in the immature murine B cell line, WEHI 231. We have determined that TGF-β rapidly induces apoptosis in a time- and dose-dependent manner. Coincident with the onset of DNA fragmentation is the cleavage of αII-spectrin into 150-, 115-, and 110-kDa fragments. We also demonstrate that the broad spectrum caspase inhibitor, Boc-D-fmk (BD-fmk), completely blocks TGF-β-mediated apoptosis and αII-spectrin cleavage, whereas specific caspase inhibitors are unable to prevent apoptosis. Our data also demonstrate...
that αII-spectrin cleavage is independent of caspase 3 and calpain activation. Finally, our results in vivo and in vitro suggest that αII-spectrin fragments generated during TGF-β-induced apoptosis may lead to the identification of novel caspase cleavage sites.

EXPERIMENTAL PROCEDURES

Materials—Caspase inhibitors were obtained from Enzyme Products and resuspended in Me₂SO. TGF-β was a generous gift of Genzyme, Inc., and was diluted in 4 mM HC1, 0.5 mg/ml bovine serum albumin, which was used as the control/carrier. αII-spectrin monoclonal antibody (mAB1622) was purchased from Chemicon, Inc. Caspase 3 polyclonal antibody (CSP3) was a gift from Idun Pharmaceuticals. Apoptosis-activating α-Fas, clone CH-11, was purchased from Upstate Biotechnology. EDTA-free protease inhibitor tablets were purchased from Roche Molecular Biochemicals. Calpain inhibitor I (ALLN), calpain inhibitor II (ALLM), and PARG monoclonal antibody (AM30) were purchased from Calbiochem. Rabbit reticulocyte lysate kits were purchased from Promega. Immobilon P was purchased from Millipore. Renaissance chemiluminescence reagent was purchased from NEN Life Science Products and used according to the manufacturer's instructions.

Cell Lines—Mouse AKR2B fibroblasts and rat FAO hepatoma cells were grown in Dulbecco's modified Eagle's medium/F-12 containing 10% fetal bovine serum. The B cell lines WEHI 231, CH33, and CH12 were cultured at 5 × 10⁵ cells/ml in Dulbecco's modified Eagle's medium/F-12 containing 10% fetal bovine serum and 30 μM 2-mercaptoethanol. Human Jurkat T cell lymphomas were cultured at 5 × 10⁶ cells/ml in Dulbecco's modified Eagle's medium/F-12 containing 10% fetal bovine serum. Cells were cultured in the presence of penicillin, streptomycin, and amphotericin B (Life Technologies, Inc.) at 37 °C and 95% O₂,5% CO₂.

Apoptosis Assay—Apoptosis was determined by the presence of an oligonucleosomal ladder in agarose gels. Briefly, 1 × 10⁶ cells were centrifuged at 1000 rpm for 5 min, washed in 1× phosphate-buffered saline, and lysed in HL buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA, and 0.1% Triton X-100) for 15 min at room temperature (10). The lysate was extracted with an equal volume of phenol and then phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated 18 h at 20 °C with an equal volume of isopropanol and 0.1 volume of 5 × NaCl. The precipitated DNA was resuspended in Tris/EDTA, pH 8.0, containing DNase-free RNase A and incubated at 37 °C for 30 min. The DNA was separated on a 1.2% agarose gel in 1× TBE and poststained with ethidium bromide. Caspase inhibitors were added at the indicated concentrations 1 h before the addition of TGF-β. Viability and cell number were determined by trypan blue exclusion.

In Vitro Translation and Cleavage Assay—Whole cell lysates were prepared by resuspension in lysis buffer (20 mM Tris, pH 7.4, 1% Triton X-100, 10% glycerol), sonicated, and centrifuged at 14,000 rpm at 4 °C for 15 min to clear cellular debris. In vitro translation was performed using T7 RNA polymerase as per the manufacturer's instructions. Briefly, 1 μg of specific regions of the αII-spectrin gene in pcDNA, αI 9–12 (repeat units 9–12) or αII 13-C (repeat 13 to C terminus) (22), were incubated with 40 μCi of [³⁵S]methionine at 30 °C for 2 h. In vitro cleavage assays were performed by incubating treated or control clarified lysates (30 μg) with 3 μl of in vitro translated αII-spectrin gene product in cleavage assay buffer (20 mM Hepes, pH 7.4, 0.5 mM Nonidet P-40, 100 mM NaCl, and 20 mM dithiothreitol) for 1 h at 37 °C. The reaction was terminated by the addition of 2× Laemmli buffer, and proteins were separated by SDS-PAGE and fluorographed before autoradiography.

Western Blotting—Western blotting was performed as described previously (10, 40). Briefly 50–100 μg of whole cell lysate were separated by SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membrane. Protein transfer was empirically determined by staining with 1.0% Ponceau S in 5% glacial acetic acid for 1 min followed by several water washes (10). The membrane was incubated for 1 h in blocking buffer (60 mM Tris, 200 mM NaCl with 0.05% Tween 20 containing 5% nonfat dry milk, pH 7.3). The membrane was subsequently incubated with a 1/1000 dilution of primary antibody in blocking buffer and then with secondary antibody. Primary antibodies included monoclonal (mAB1622) or polyclonal (pAb) antibodies to αII-spectrin (41), α-bdp-150 polyclonal antibody to calpain-specific αII-spectrin cleavage fragment (42), or PARP monoclonal antibody (43). The blot was washed extensively and incubated with a 1/2000 dilution of rabbit α-mouse-horseradish peroxidase (Accurate Antibodies), goat α-rabbit- horseradish peroxidase (Transduction Laboratories), or rabbit α-goat-horseradish peroxidase (Accurate Antibodies) for 1 h and processed using the Renaissance chemiluminescence reagent according to manufacturer's directions.

RESULTS

We have previously demonstrated that TGF-β induces apoptosis, which can be distinguished from growth arrest using the broad spectrum caspase inhibitor, BD-fmk, in the WEHI 231 cell line (10). To further examine the apoptotic process, we have identified αII-spectrin as a substrate for cleavage. Western blot analysis using a monoclonal antibody revealed that the endogenous 240-kDa αII-spectrin is cleaved into 150-, 115-, and 110-kDa fragments after treatment with 5 ng/ml TGF-β for 24 h (Fig. 1). TGF-β can also induce the cleavage of αII-spectrin during apoptosis in another immature mouse B cell line (CH33), a mature mouse B cell line (CH12), and a rat hepatoma (FAO) cell line (data not shown, Fig. 1). TGF-β treatment of the mouse AKR2B fibroblast cell line for 24 h did not induce apoptosis (data not shown) and did not induce cleavage of αII-spectrin (Fig. 1). We have previously reported that the broad spectrum caspase inhibitor, BD-fmk (BD), dose responsively inhibits TGF-β-induced DNA ladder formation in WEHI 231 cells (10). The addition of BD-fmk was also able to prevent the cleavage of αII-spectrin into the 115- and 110-kDa fragments but could not prevent generation of the 150-kDa fragment (Fig. 1). The 150-kDa fragment may be generated by different proteases and does not appear to be caspase-specific (22, 36). These results suggest that the 115- and 110-kDa αII-spectrin fragments generated during TGF-β-mediated apoptosis are caspase-dependent.

To determine the onset of αII-spectrin cleavage in WEHI 231 cells, cellular lysates were analyzed by Western blotting at several time points after TGF-β administration. TGF-β induced a time-dependent cleavage of αII-spectrin into the caspase-dependent 115- and 110-kDa fragments as early as 8 h after the addition (Fig. 2). DNA was analyzed at several time points to determine the time required for initiation of DNA ladder formation and whether it coincides with αII-spectrin cleavage after TGF-β administration. TGF-β rapidly induced the 180-base pair oligonucleosomal DNA ladder in WEHI 231 cells as early as 8 h after administration (Fig. 3). Increased DNA ladder formation corresponded with decreased cellular number and increased terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL)-positive cells (Ref. 10; data not shown). These data indicate that TGF-β induces cleavage of

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αII-spectrin coincident with the apoptosis process.

The results of Fig. 1 and our previous results (10) suggest that a caspase protease may be responsible for mediating αII-spectrin cleavage and apoptosis in response to TGF-β in WEHI 231 cells. To date, cleavage of αII-spectrin has only been shown to occur via calpain, caspase 3 (Cpp32, Yama), or caspase 3-like PARP-sensitive proteases (22, 35, 37–39). To determine if one of these three proteases may be responsible for TGF-β-mediated αII-spectrin cleavage in WEHI 231 cells, peptide inhibitors were used (Fig. 4A). The addition of TGF-β generated the characteristic 150-, 115-, and 110-kDa αII-spectrin fragments. Incubation with the broad spectrum caspase inhibitor, BD-fmk, completely abolished the generation of the 115- and 110-kDa products. DEVD-fmk was only slightly able to reduce the 110-kDa fragment but had no effect on the 115-kDa product. The 150-kDa fragment was not blocked by BD-fmk or DEVD-fmk and was elevated compared with the control. The suggestion that the proteolytic enzymes responsible for αII-spectrin cleavage are caspases is supported by the essential P1 aspartate requirement demonstrated by BD-fmk inhibition of the 115- and 110-kDa fragments and the inability of the cognate negative control FA-fmk to prevent cleavage (Fig. 4A). Calpain inhibitor I (CpI) was unable to block αII-spectrin cleavage in the presence of TGF-β for 24 h (Fig. 4A). The addition of calpain inhibitor appeared to significantly increase the amount of αII-spectrin cleavage that occurred at 24 h and resulted in the complete disappearance of the 150-kDa fragment. Calpain inhibitor II (CpII) was also unable to block TGF-β-induced αII-spectrin cleavage (data not shown). Neither CpI or CpII were able to prevent apoptosis induced by TGF-β (data not shown). Thus TGF-β-mediated cleavage of αII-spectrin does not appear to occur via activation of calpain in this system. To confirm that

αII-spectrin induction is caspase 3-independent

The caspase 3 inhibitor, DEVD-fmk, could not prevent the TGF-β-mediated generation of the 115- and 110-kDa αII-spectrin fragments (Fig. 4A). At concentrations as high as 100 μM, DEVD-fmk reduced but was unable to prevent αII-spectrin cleavage induced by TGF-β. DEVD-fmk was also unable to prevent TGF-β-induced DNA ladder formation at several concentrations evaluated up to 100 μM or at earlier time points (data not shown). The partial reduction of the 110-kDa fragment by DEVD-fmk may be due to the high concentrations used, permitting interaction with other caspases. Lower concentrations (10–30 μM) of DEVD-fmk did not cause a reduction in the 110-kDa αII-spectrin fragment (data not shown). Recent reports indicate that a DEVD-insensitive caspase 3-like protease is also able to cleave αII-spectrin into 150- and 120-kDa fragments when activated by α-Fas in Jurkat T cells (22, 35, 37–39).
This caspase 3-like protease is capable of cleaving αII-spectrin as well as the DNA repair enzyme PARP (37, 39). To determine if a caspase 3-like activity might be present in WEHI 231 cells, we determined if PARP was cleaved from an intact 116-kDa protein into the characteristic 85-kDa fragment (39) in the presence of TGF-β (Fig. 4C). The results indicate that PARP is not cleaved during TGF-β-induced apoptosis even after 24 h; however, actinomycin D treatment induced the characteristic cleavage of PARP and was prevented by DEVD-fmk in WEHI 231 cells. Jurkat cells treated with anti-Fas also induced PARP cleavage that could be also blocked by DEVD-fmk (Fig. 4C, data not shown.) Taken together the data suggest that αII-spectrin cleavage in WEHI 231 cells does not occur via previously reported proteases.

Although inhibitor studies and alternate protein substrates provide strong evidence that TGF-β-mediated αII-spectrin cleavage and apoptosis are not mediated via caspase 3 or caspase 3-like proteases, it remains possible that a novel caspase 3 family member or a novel cleavage site in αII-spectrin is present in which traditional inhibitors (DEVD-fmk) and alternate substrates (PARP) are ineffective or unaltered. To evaluate the possibility that caspase 3 or a caspase 3-like protease may be present or activated in WEHI 231 cells, Western blot analysis was performed using a caspase 3 polyclonal antibody (Fig. 5A). This antibody recognizes the uncleaved inactive proform (32 kDa) as well as the activated 20- and 10-kDa subunits of caspase 3 (41). WEHI 231 cells produce inactive caspase 3 (Fig. 5, A and B); however, TGF-β addition does not activate this protease as evidenced by the absence of 20- and 10-kDa products. Jurkat cells treated with activating α-Fas antibody for 4 h also activated caspase 3/caspase 3-like protease (Fig. 5A). Actinomycin D and cycloheximide treatment of WEHI 231 cells cleaved caspase 3 to the characteristic 20-kD and 10-kDa subunits (Fig. 5B). Incubation with DEVD-fmk and BD-fmk blocked the α-Fas-induced formation of the active 20- and 10-kDa caspase 3 subunits in Jurkats (Fig. 5A). DEVD was also able to block caspase 3 activation in WEHI-231 cells treated with actinomycin D by preventing the reduction (i.e. cleavage) of the 32-kDa pro-caspase 3 (Fig. 5C). Although inactive caspase 3 was detected in WEHI 231 cells, the amount of pro-caspase 3 enzyme (32 kDa) was qualitatively lower. BD-fmk dose responsively inhibited DNA fragmentation, whereas DEVD-fmk was without effect even at high (100 μM) concentrations in WEHI 231 cells (10). DEVD-fmk also effectively prevented DNA ladder formation in response to α-Fas in Jurkat cells (data not shown). These results indicate that TGF-β-induced αII-spectrin cleavage, as well as apoptosis, occurs through a caspase 3-independent mechanism in WEHI 231 cells.

To further distinguish TGF-β-mediated αII-spectrin cleavage from caspase 3-mediated cleavage, α-Fas-treated Jurkat cells or TGF-β-induced WEHI 231 cell lysates were compared to analyze the molecular weights of the αII-spectrin fragments (Fig. 6). The data indicate that the αII-spectrin cleavage fragments generated in response to α-Fas antibody in Jurkat cells and those generated in WEHI 231 in response to TGF-β are of different molecular weights (Fig. 6). The apparent molecular weights of the large and small fragments in Jurkat cells are larger than those generated in WEHI 231 cells. In WEHI 231 cells, both αII-spectrin cleavage species are blocked by the general caspase inhibitor BD-fmk, whereas in α-Fas-treated Jurkat cells, an intermediate cleavage fragment is detected in the presence of BD-fmk (Fig. 6). Identical results were obtained using an αII-spectrin specific polyclonal antibody, Raf A, instead of the monoclonal (mAb1622) antibody (Ref. 44; data not shown).

**Fig. 5.** A, anti-Fas activates caspase 3. WEHI 231 cells were treated with carrier (C), 5 ng/ml TGF-β for 24 h (TGF-β), or 5 ng/ml TGF-β for 24 h after a 1-h preincubation with 100 μM BD-fmk (β/BD) or DEVD-fmk (β/DEVD). Jurkat cells were treated with carrier (C), 100 ng/ml anti-Fas (α-Fas) for 4 h, or 100 ng/ml anti-Fas (α-Fas) for 4 h after a 1-h preincubation with 100 μM BD-fmk (α-Fas/BD) or DEVD-fmk (α-Fas/DEVD). Whole cell lysates were collected, separated on a 12.5% SDS-PAGE gel, and analyzed by Western blotting using the caspase 3 polyclonal antibody (CSP3) as described under "Experimental Procedures." B, WEHI 231 cells contain but do not activate caspase 3. WEHI 231 cells were treated with carrier (C), 5 ng/ml TGF-β for 24 h (TGF-β), 1 μg/ml actinomycin D for 4 h, or 10 μg/ml cycloheximide (CHX) for 4 h. Jurkat cells were treated with carrier (C) or 100 ng/ml anti-Fas (α-Fas) for 4 h. Whole cell lysates were collected, separated on a 12.5% SDS-PAGE gel, and analyzed by Western blotting using the caspase 3 polyclonal antibody (CSP3) as described under "Experimental Procedures." C, caspase 3 activation in WEHI 231 cells. WEHI 231 cells were treated with carrier (C), 5 ng/ml TGF-β (20 h), 1 μg/ml actinomycin D for 4 h (Act.), or 1 μg/ml actinomycin D for 4 h after a 1-h preincubation with 100 μM DEVD-fmk (Act./DEVD). Whole cell lysates were collected, separated on a 12.5% SDS-PAGE gel, and analyzed by Western blotting using the caspase 3 polyclonal antibody (CSP3) as described under "Experimental Procedures." P20, 20-kDa subunit; P10, 10-kDa subunit.

In vivo analysis (Fig. 6) indicated that αII-spectrin cleavage fragments generated by TGF-β were of molecular weights distinct from those generated in Jurkat cells when treated with caspase 3-activating α-Fas. The molecular weights of the TGF-β-stimulated αII-spectrin fragments were also distinct from the fragments generated by several other caspases in vitro (22). To determine if in vitro cleavage assays using WEHI 231 lysates would provide similar molecular weight differences in αII-spec-
fragments of 150, 115, and 110 kDa. This is the first report to
middle third and covers 430 amino acids of the
optosis in any system. Our data also provide evidence that
fmk blocked both apoptosis and PARP cleavage but only par-
cedures” and subjected to
in vitro
was
cleavage products generated
in elucidating the actual cleavage site(s).
and that such assays will continue to provide a useful tool
vivo
correspond to similar regions within the intact protein, sug-
II-spectrin cleavage during apoptosis (37–39). MCF7 breast
II-spectrin gene in pcDNA3, designated
II-spectrin, in the mouse
II-spectrin and PARP but with differential
cleavage of a novel substrate,
II-spectrin cleavage
II-spectrin cleavage pattern
in vivo. Jurkat cells were treated with carrier (C), 100 ng/ml α-Fas
(a-Fas), or 100 ng/ml α-Fas (α-Fas/BD) for 4 h after a 1-h preincubation
with 100 μM BD-fmk. WEHI 231 cells were treated with carrier (C), 5
ng/ml TGF-β (TGF-β), or 5 ng/ml TGF-β (TGF-β/BD) for 24 h after a
1-h preincubation with 100 μM BD-fmk. Whole cell lysates were col-
collected, separated on an 8% SDS-PAGE gel, and analyzed by Western
blotting using the αII-spectrin monoclonal antibody as described under
“Experimental Procedures.” 

29 kDa
26 kDa
19 kDa

Fig. 6. TGF-β induces a distinct αII-spectrin cleavage pattern
in vivo. Jurkat cells were treated with carrier (C), 100 ng/ml α-Fas
(a-Fas), or 100 ng/ml α-Fas (α-Fas/BD) for 4 h after a 1-h preincubation
with 100 μM BD-fmk. WEHI 231 cells were treated with carrier (C), 5
ng/ml TGF-β (TGF-β), or 5 ng/ml TGF-β (TGF-β/BD) for 24 h after a
1-h preincubation with 100 μM BD-fmk. Whole cell lysates were col-
collected, separated on an 8% SDS-PAGE gel, and analyzed by Western
blotting using the αII-spectrin monoclonal antibody as described under
“Experimental Procedures.” 

DISCUSSION
We have demonstrated that TGF-β rapidly induces apoptosis
and cleavage of a novel substrate, αII-spectrin, in the mouse
immature B cell line, WEHI 231. Induction of apoptosis by
TGF-β as detected by DNA ladder formation is simultaneously
accompanied by cleavage of intact αII-spectrin (240 kDa) into
fragments of 150, 115, and 110 kDa. This is the first report to
identify αII-spectrin as a substrate during TGF-β-induced apop-
tosis in any system. Our data also provide evidence that
αII-spectrin in WEHI 231 cells is cleaved by a protease other
than those previously reported to cleave αII-spectrin during
apoptosis (35, 37–39) and suggests that a novel mechanism of
αII-spectrin cleavage may occur in WEHI 231 cells. Activation
of caspase 3 by actinomycin D or cycloheximide but not TGF-β
indicates that at least two distinct apoptotic pathways exist
in WEHI 231 cells. Finally, analysis of the estimated αII-spectrin
cleavage products generated in vivo and in vitro suggest that
the potential cleavage recognition sites in αII-spectrin may also
be unique.

Recent reports have suggested that caspase 3 is required for
αII-spectrin cleavage during apoptosis (37–39). MCF7 breast
cancer cells lacking caspase 3 are unable to cleave αII-spectrin
(37). In Jurkat cells, caspase 3-like proteases have been re-
ported to cleave αII-spectrin and PARP but with differential
sensitivity to the caspase 3 inhibitor, DEVD-fmk (39). DEVD-
fmk blocked both apoptosis and PARP cleavage but only par-
tially inhibited αII-spectrin cleavage. In neuroblastoma cells,
treatment with staurosporine induced cleavage of αII-spectrin
at both caspase 3 and calpain cleavage sites (22). However, in
all of these examples, activation of caspase 3 or a caspase 3-like
protease participated in the cleavage of αII-spectrin. Our data
demonstrate that cleavage of αII-spectrin in TGF-β-treated
WEHI 231 cells is caspase 3-independent based on several lines
of evidence. 1) In vivo generation of αII-spectrin cleavage prod-
ucts in TGF-β-treated WEHI 231 cells are smaller than in
α-Fas-treated Jurkat cells as shown in Fig. 6 and by other
groups (35, 37–39). 2) Although BD-fmk effectively blocks apop-
tosis and αII-spectrin cleavage, the caspase 3 inhibitor,
DEVD-fmk, is ineffective in blocking TGF-β-induced apoptosis
or αII-spectrin cleavage. 3) Cleavage of a known caspase 3 and
caspase 3-like substrate, PARP, does not occur in WEHI 231
cells during TGF-β-induced apoptosis. 4) In WEHI 231 cells,
caspase 3 is present in its pro-enzyme form (32 kDa) and can be
activated by actinomycin D or cycloheximide but not by TGF-β
during the induction of αII-spectrin cleavage or apoptosis. This
is the first known report of caspase 3 activation in WEHI 231
cells and suggests that although more than one apoptotic path-
way exists and is activated, specific caspase activation is de-
pendent upon the initiating stimulus. The data are consistent
with previous reports of actinomycin D-inducible activation of
caspase 3 and apoptosis in human leukemia cell lines (45, 46).

5) Analogous to the in vivo situation, cleavage of αII-spectrin in
vitro with TGF-β-treated WEHI 231 lysates results in the
generation of products that are different in molecular weight
from α-Fas/caspase 3-mediated cleavage of αII-spectrin with
Jurkat cellular lysates. The 150-kDa product produced in Jur-
kat cells has previously been reported to occur at a caspase-
-independent hypersensitive site (22, 36) and is consistent with
the 150-kDa caspase-independent fragment we detect in WEHI
231 cells in response to TGF-β.

α-Fas generated in vivo cleavage of αII-spectrin in Jurkat
cells produces a predominant 120-kDa fragment as well as a
minor 112-kDa product. The 120-kDa fragment is consistent
with a previously reported caspase 3-mediated DETD cleavage
site within the protein (22, 38). In vitro cleavage assays using
α-Fas-treated Jurkat lysates identified cleavage sites at DETD
and DSLD (22). The in vitro generated DETD site is consistent
with the in vivo aII-spectrin cleavage site, whereas the DSLD site may be consistent with the 112-kDa product we routinely observe in vivo in Jurkats (35, 37, 38). This 112-kDa aII-spectrin product has previously been observed in vivo in several other systems; however, the origin of this product has not been determined (34, 35, 37–39).

Analysis of the in vivo and in vitro aII-spectrin fragments generated during TGF-β-induced apoptosis in WEHI 231 cells has allowed us to determine potential sites of cleavage based on molecular weight. In vivo, TGF-β generates aII-spectrin fragments of 115 and 110 kDa. The sizes of the major aII-spectrin cleavage products (115 and 110 kDa) generated in the presence of TGF-β addition in WEHI 231 cells are distinct from the aII-spectrin fragments generated in vitro by caspases 1, 2, 3, 4, 6, or 7 (22). These molecular weights correspond with potential unique caspase cleavage sites, EVND and EQID, present within intact aII-spectrin. Interestingly, the potential EXXD sites would represent a similar but distinct protease substrate recognition sequence. The alteration in the P4 amino acid from aspartate (caspase 3 family) to glutamate would maintain charge specificity while perhaps requiring altered substrate conformation due to the additional -CH2 group present in the glutamate backbone. Other potential sites within this region include KXXD sequences, which are also prominent and interspersed throughout aII-spectrin. The number of these potential KXXD sites within aII-spectrin would suggest that other cleavage products should be present; however, we were unable to detect products other than those described. Analysis of these and other potential TGF-β-mediated aII-spectrin cleavage sites, as well as new substrates, are currently under active investigation. Definitive proof, however, will require demonstrating that aII-spectrin is cleaved after aspartate residues, which is consistent with the predicted sites.

TGF-β has been shown to induce apoptosis in other systems, in particular primary hepatocytes and hepatoma cells (18, 23, 24, 27, 28, 47). In these cells, apoptosis is blocked by the caspase inhibitor, ZVAD-fmk (27, 28, 31, 47). In rat hepatoma FAO cells, TGF-β has been shown to induce apoptosis via caspase 2 and cleave U1–70-kDa protein (27). In addition, TGF-β-induced apoptosis in human Hep3B cells is also inhibited by ZVAD-fmk and cleaves the catalytic subunit of DNA protein kinase, DNA-PKCs (47). In WEHI 231 cells, however, neither U1–70-kDa nor DNA-PKCs is cleaved, and up to 200 μM ZVAD-fmk as well as tetrapeptide inhibitors of known caspases, including caspase 2, do not block apoptosis mediated by TGF-β (10). The distinct mechanisms of apoptosis in related systems suggests that alternate and perhaps cell-type-specific pathways exist.

Our data indicate that TGF-β-mediated apoptosis occurs via an aspartyl protease that can only be inhibited by the broad spectrum caspase inhibitor, BD-fmk. Indications that this aspartyl protease is a caspase are based on several lines of evidence. The inhibitory mechanism of BD-fmk is based on a single amino acid, aspartate, conjugated to a cell-permeable, irreversible fluoromethyl ketone. Aspartyl-cleaving proteases are the only proteases so far shown to be involved in apoptotic pathways. Incubation with BD-fmk should bind to and inhibit caspase (aspartyl protease) activation that occurs at aspartate residues. In previous work, we have shown that an inhibitor of non-aspartyl-activating cysteine proteases, FA-fmk, used as a negative control, failed to prevent TGF-β-mediated apoptosis or aII-spectrin cleavage, demonstrating the essential P4 specificity for aspartate. The requirement for aspartate at the P4 cleavage position is a unique and identifying characteristic of all identified caspases (10). We have also reported that the aspartyl-cleaving serine protease inhibitor, AAD-fmk, was unable to affect TGF-β-induced apoptosis, suggesting a specificity for aspartyl cysteine proteases as mediators of apoptosis (10). Generation of a 180-base pair oligonucleosomal DNA ladder has only been shown to be generated by aspartyl proteases (18, 48), and failure of more specific tetrapeptide caspase inhibitors to block apoptosis or aII-spectrin cleavage mediated by TGF-β suggests several possibilities that could account for the observed protease activity. These might include activation of a known caspase with altered substrate cleavage sites, failure of the tetrapeptide inhibitors to recognize existing caspases, an unknown nonaspartyl protease inhibitory function for BD-fmk, or a novel caspase capable of mediating apoptosis in the presence of TGF-β.

In summary our data provide the first report that TGF-β stimulates the cleavage of aII-spectrin coincident with the induction of apoptosis. In addition we have shown that aII-spectrin cleavage after TGF-β treatment is independent of the known aII-spectrin-cleaving proteases; calpain, caspase 3, or caspase 3-like proteases. Examination of potential cleavage sites in aII-spectrin suggests that proteolysis may occur via novel recognition sequences in WEHI 231 after exposure to TGF-β. Taken together, our results suggest that TGF-β-mediated apoptosis in WEHI 231 cells represents a physiologically relevant system that may occur via a specific mechanism(s) not previously identified in other systems.

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