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Insulin-Like Growth Factor-Binding Protein 3 Induces Caspase-Dependent Apoptosis through a Death Receptor-Mediated Pathway in MCF-7 Human Breast Cancer Cells

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ABSTRACT

Insulin-like growth factor-binding protein (IGFBP)-3 has been shown to potently inhibit cell proliferation in various cell systems. However, the specific mechanisms involved in the antiproliferative action of IGFBP-3 have yet to be elucidated. In the present study, we demonstrate that IGFBP-3 induces apoptosis in an insulin-like growth factor (IGF)-independent manner through the activation of caspases involved in a death receptor-mediated pathway in MCF-7 human breast cancer cells. Induction of IGFBP-3 using an ecdysone-inducible expression system inhibited DNA synthesis in an IGF-IGF receptor axis-independent fashion and resulted in the subsequent induction of apoptosis and an increase in caspase activity. Similar results were obtained when cells were transfected with GGG-IGFBP-3, an IGFBP-3 mutant unable to bind IGFs, corroborating the IGF-independent action of IGFBP-3. Additional caspase activity studies and immunoblot analyses using specific caspase substrates and/or caspase inhibitors revealed that the growth-inhibitory effect of IGFBP-3 results mainly from its induction of apoptosis (in particular, activation of caspase-8 and -7). Analyses of caspase-9 activity and release of cytochrome *c* into the cytosol confirmed that the mitochondria-mediated pathway is not involved. Taken together, these results show that IGFBP-3 expression leads to the induction of apoptosis through the activation of caspases involved in a death receptor-mediated pathway and that IGFBP-3 functions as a negative regulator of breast cancer cell growth, independent of the IGF-IGF receptor axis.

INTRODUCTION

The insulin-like growth factor-binding proteins (IGFBPs) are components of the insulin-like growth factor (IGF) axis, and their superfamily comprises six high-affinity species (IGFBPs 1–6) and several low-affinity binders [IGFBP-related proteins (1–5)]. The classical role of the IGFBPs involves IGF binding and modulation of IGF signaling; however, recent data suggest that some IGFBPs may play a more active, IGF-independent role in growth regulation of various cell systems (6–19). In particular, IGFBP-3 has been shown to potently inhibit the proliferation of various cell types in an IGF-independent manner. The concept of IGF-independent actions for IGFBP-3 has been supported by the demonstration that (*a*) exogenous IGFBP-3 binds to specific proteins on the cell surface, and this interaction is strongly correlated with the ability of IGFBP-3 to inhibit cell growth (8, 9); (*b*) overexpression of human IGFBP-3 cDNA inhibits cell proliferation (10–12); (*c*) IGFBP-3 mediates transforming growth factor β (13)-, retinoic acid (14)-, antiestrogen (15)-, vitamin D analog (16)-, and tumor necrosis factor (TNF)-α (17)-induced growth inhibition; (*d*) regulation of IGFBP-3 gene expression plays a role in signaling by p53, a potent tumor suppressor protein (18); and (*e*) IGFBP-3 fragments inhibit the stimulation of DNA synthesis induced by either IGF-I or insulin (19). Recently, several reports have demonstrated that IGFBP-3 induces apoptosis in PC-3 prostate cancer (20) and MCF-7 breast cancer cells (21) and enhances ceramide-induced apoptosis in Hs578T breast cancer cells (22). These apoptosis-inducing effects of IGFBP-3 may be due to sequestering of IGFs, thus blocking their antiapoptotic activities (21). Alternatively, IGFBP-3 may act via IGF-independent pathways because IGFBP-3 can induce apoptosis in an IGF receptor-negative mouse fibroblast cell line (20) and can accentuate apoptosis under conditions where IGF-I does not elicit a survival effect (22). More direct evidence comes from the demonstration that IGFBP-3 mutants that do not bind IGFs stimulate apoptosis in human prostate cancer cells (23). Additional studies by Butt *et al.* (24) have demonstrated that IGFBP-3 can modulate the expression of the Bax:Bcl-2 protein ratio in apoptotic human breast cancer cells in a p53-independent manner. In addition, caspase-3 activity, as well as IGFBP-3 levels, is increased in Hs578T breast cancer cells undergoing apoptosis in response to the chemotherapeutic agent paclitaxel (25). Despite clear evidence of IGFBP-3's activation of apoptosis in an IGF-independent fashion, the mechanisms by which these effects are achieved have yet to be elucidated.

Programmed cell death plays a crucial role in normal development, defense of homeostasis, and pathological situations (26–28). Apoptotic signals are generally believed to be mediated through a caspase system controlled by two autonomous and interacting pathways that are associated with either cell surface death receptors or mitochondria. Apoptotic stimuli such as DNA-damaging agents trigger the release of mitochondrial cytochrome *c* into the cytosol, which controls the assembly of an apoptosome composed of Apaf-1 (apoptotic protease-activating factor 1), Bcl-X_L, and procaspase-9 (29). Binding of cytochrome *c* and dATP to Apaf-1 results in the activation of caspase-9 and the subsequent activation of downstream caspases such as caspase-3, which kill cells by cleaving key intracellular proteins, whereas Bcl-X_L presumably inhibits this function. In contrast, binding of death ligands to their death receptors leads to the recruitment of a variety of death domain-bearing adapter proteins, which in turn recruit the most upstream caspases, such as caspase-8 and -10, an event that results in the activation of these caspases and downstream effector caspases. In addition, these activated upstream caspases may act on the mitochondria to facilitate cytochrome *c* release, thereby further amplifying the apoptotic signal. Here we show that IGFBP-3 inhibits cell growth in an IGF-independent manner and that its growth-inhibitory effect results from the caspase-dependent induction of apoptosis in MCF-7 human breast cancer cells. We also show that IGFBP-3 facilitates the induction of biochemical events such as the activation of the initiator caspase-8 and the processing of caspase-7 but minimal release of cytochrome *c* from mitochondria and processing of caspase-9, suggesting that IGFBP-3-induced apoptosis may be accomplished through the activation of caspases involved in a death receptor-mediated pathway in MCF-7 human breast cancer cells.

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MATERIALS AND METHODS

Reagents and Kits. Tissue culture reagents and plastics were purchased from Mediatech (Hemdon, VA), Becton Dickinson (Franklin Lakes, NJ), and Nunc (Naperville, IL). Special reagents and kits were obtained from the following sources: Zeocin, Geneticin sulfate, and ponasterone A (Invitrogen, Carlsbad, CA); IGFBP-3 radioimmunoassay kit (Diagnostic Systems Laboratories, Webster, TX); CellTiter 96 cell proliferation assay (Promega, Madison, WI); TNF-α (Calbiochem, San Diego, CA); IGFBP-5 (Austral Biologicals, San Ramon, CA); annexin V apoptosis kit (Clontech, Palo Alto, CA); cell death detection ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN); aminomethylcoumarin (AMC) derivatives (caspase substrates) and cell-permeable aldehyde derivatives (caspase inhibitors) of tetrapeptide sequences that are recognized by distinct caspases [Ac-DEVD-AMC (caspase-3/-7), Ac-LEHD-AMC (caspase-9), Ac-IETD-AMC (caspase-8), and Z-VAD-fluoromethylketone (FMK) (broad-spectrum caspase inhibitor), BIOMOL Research Laboratories (Plymouth Meeting, PA); Z-IETD-FMK (caspase-8 inhibitor) and Z-DEVD-FMK (caspase-3/-7 inhibitor), BD PharMingen (San Diego, CA; Refs. 30–33)]. Antibodies against poly(ADP-ribose) polymerase (PARP), cleaved PARP, caspase-9, cleaved caspase-9, caspase-7, and caspase-10 were purchased from Cell Signaling Technology (Beverly, MA); antibody against caspase-8 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); and antibodies against cytochrome *c*, Bid, and caspase-2 were purchased from BD PharMingen. IGF-I, ^{125}I-labeled IGF-I and -II, and a monoclonal antibody against IGFBP-3 were generously provided by Diagnostic Systems Laboratories. The ecdysone-inducible expression system was purchased from Invitrogen.

Cell Cultures. The MCF-7 human breast cancer cell line and its derivatives expressing an IGFBP-3 in an inducible manner were maintained in DMEM supplemented with 4.5 g/liter glucose, 110 mg/liter sodium pyruvate, and 10% fetal bovine serum (FBS). At 60–70% confluence, the cells were washed with fresh serum-free media (SFM) and then incubated in phenol red-free SFM in the presence or absence of 15 μM ponasterone A for an additional 72 h, unless specifically indicated in the text.

Establishment of MCF-7-Derived Cell Line Expressing IGFBP-3 in an Inducible Manner. The MCF-7 human breast cancer cell was purchased from the American Type Culture Collection (Manassas, VA). A cDNA containing 1.5-kb human IGFBP-3 (equivalent to the 674-2191 cDNA sequence) was ligated into the pIND expression vector, which contains five modified ecdysone response elements. This construct was cotransfected with a second plasmid, pVgRXR, expressing the ecdysone receptor (VgEcR) and the retinoid X receptor (RXR), into MCF-7 cells using FuGENE 6 transfection reagent (Roche, Indianapolis, IN). After 48 h, the cells were split into selective medium containing G418 (800 μg/ml) for pIND/IGFBP-3 and Zeocin (100 μg/ml) for pVgRXR, and foci were picked to generate a stable cell line expressing pVgRXR and pIND/IGFBP-3. Three days after the addition of ponasterone A, which is an ecdysone analog inducing the heterodimer of RXR and VgEcR to bind to the hybrid ecdysone response element in the pIND vector, conditioned media (CM) were collected to test for ponasterone A-inducible expression of IGFBP-3. pVgRXR-transfected MCF-7 cells were used as a negative control (EcR cells).

Plasmids and Transfections. The GGG-IGFBP-3 mutant cDNA was generated by site-directed mutagenesis at residues Ile ^{56}, Leu ^{80}, and Leu ^{81} to Gly ^{56}, Gly ^{80}, and Gly ^{81}, as described previously (34). Binding studies (including BIAcore analysis) showed that the GGG-IGFBP-3 mutant protein, generated in *Escherichia coli* and baculovirus expression systems, abolished affinity for IGFs (34). For transfection, GGG-IGFBP-3 mutant cDNAs were subcloned into the pCMV6 vector as described previously (34). MCF-7 cells were seeded in DMEM supplemented with 10% FBS and transfected with the pCMV6 vector or pCMV6/GGG-IGFBP-3 using FuGENE 6 transfection reagent. Twenty-four h after transfection, the media were replaced with SFM and maintained as indicated above.

[^{3}H]Thymidine Incorporation Assay. Cells were grown in 24-well plates until 60–70% confluent, when the media were replaced with phenol red-free SFM in the presence or absence of 15 μM ponasterone A. After 68 h, 0.1 μCi of [^{3}H]thymidine (25 Ci/mM; New England Nuclear, Boston, MA) in a volume of 25 μl was added to each well for a 4-h pulse. The rate of DNA synthesis was estimated by measuring the trichloroacetic acid-precipitable radioactivity, as described previously (35).

Cell Proliferation Assay. Cells were grown in 96-well plates until 60–70% confluent, when the media were replaced with phenol red-free SFM in the presence or absence of 15 μM ponasterone A for 72 h. Assays were performed by adding 100 μl of CellTiter 96 Aqueous one solution (1:5 dilution with phenol red-free SFM) directly to culture wells after aspirating media and incubating for 1 h at 37°C. The quantity of viable cells was measured in a 96-well plate reader at 490 nm.

Flow Cytometry and Apoptosis Assay. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Both attached and floating cells were harvested by trypsinization, pelleted at 1000 rpm for 5 min, and washed three times with PBS. Each sample of cells was resuspended in a propidium iodide staining solution (50 μg/ml propidium iodide, 100 units/ml RNase A, 0.1% Triton X-100, and 0.1% sodium azide in PBS) and shaken for 30 min in the dark before measurement on a FACSCalibur flow cytometer (Becton Dickinson) using an argon laser at 488 nm for excitation. Apoptotic cells were detected using a FITC-labeled annexin V binding assay according to the manufacturer's directions. All data were analyzed using the Cell Quest software package (Becton Dickinson).

Cell Death Detection ELISA Assay. The photometric cell death detection ELISA is based on quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively. In brief, equal numbers of cells (1×10^4 cells/ml) were plated on 24-well culture plates in 10% FBS-supplemented DMEM. At 60–70% confluence, cells were treated with 15 μM ponasterone A in the absence or presence of 25 μM Z-VAD-FMK or 20 μM Z-IETD-FMK in phenol red-free SFM for 72 h. TNF-α (20 ng/ml) and 10% FBS were used as positive and negative controls, respectively. Adherent and floating cells were used to prepare the cytosolic fractions according to the vendor's instructions. Equal volumes of the cytosolic fractions were transferred to the streptavidin-coated microtiter plates and incubated with anti-histone-biotin and peroxidase-conjugated anti-DNA immunoreagents. The reaction products were read using a Bio-Rad microplate reader. Each experiment was carried out with duplicate samples and repeated at least three times.

Measurement of Caspase Activity. Caspase activity was measured in lysates by analyzing the *in vitro* hydrolysis of Ac-DEVD-AMC (caspase-3 and -7), Ac-IETD-AMC (caspase-8), Ac-LEHD-AMC (caspase-9), or a mixture of Ac-DEVD-AMC and Ac-LEHD-AMC (total caspases; Refs. 30–33). In brief, cells were seeded on 12-well plates, cultured until 60–70% confluent, and then incubated in phenol red-free SFM with or without ponasterone A or other reagents for the times indicated. Cells from each well were lysed in 100 μl of ice-cold lysis buffer [50 mM HEPES (pH 7.4), 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 0.1% Triton X-100, 1 mM DTT, and 0.1 mM EDTA]. Fluorogenic peptide substrates were added to each well of a fluorometer plate to a final concentration of 40 μM in the assay buffer [50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 10 mM DTT, 1 mM EDTA, and 10% glycerol]. The fluorescence of the AMC released from the substrate was measured in a kinetic mode for up to 16 h using a Bio-Rad Fluoromark Reader with the excitation and emission wavelength set at 390 and 460 nm, respectively. Data were analyzed using Microplate Manager III Macintosh Data Analysis software (Bio-Rad, Melville, NY). Enzyme activity was quantitated against a standard fluorescence curve generated using AMC over a concentration range of 0–1000 nM.

Preparation of Cell Lysates for Western Blots. Cell lysates were prepared as described previously, with minor modification (36). In brief, confluent cells were washed with cold PBS and then scraped from plates in the presence of cold lysis buffer containing 20 mM Tris (pH 8.0), 1% NP40, 0.1% SDS, 150 mM NaCl, 0.5% sodium deoxycholate, and Complete Protease Inhibitor mixture (Boehringer Mannheim). Floating cells in the CM were collected by centrifugation and resuspended in cold lysis buffer. Cell lysates were rocked for 15 min at 4°C in a microcentrifuge tube and centrifuged to remove cell debris. The aliquots were stored at −70°C until further use.

Preparation of Cytosolic and Mitochondrial Extracts. For the detection of cytochrome *c*, cytosolic and mitochondrial extracts were prepared as described previously (37). In brief, cells (2×10^6) were harvested by scraping directly in the medium, washed in cold PBS, and permeabilized for 1 min at room temperature in isotonic sucrose buffer [250 mM sucrose, 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and 1 mM EDTA (pH 7.1)] containing 0.025% digitonin and Complete Protease Inhibitor mixture. Digitonin was used to selectively permeabilize the plasma membranes of cells to obtain the cytosolic fractions. Cells were centrifuged at 4°C ($15{,}000 \times g$ for 10 min), and the supernatant was designated the cytosolic fraction. The pellet of permeabilized cells was incubated for 10 min on ice in lysis buffer containing 0.5% Triton X-100 and Complete Protease Inhibitor mixture to release membrane- and organelle-bound soluble proteins including mitochondrial cytochrome *c*. The Triton X-100-soluble (mitochondrial) fraction was separated by centrifugation at $15{,}000 \times g$ for 10 min, and all fractions were stored at −70°C for Western blot analysis.

Western Blot Analysis. The CM from IGFBP-3-induced cells were size-fractionated by 12–15% SDS-PAGE under nonreducing conditions, whereas cell lysates were size-fractionated under reducing conditions. Size-fractionated proteins were electrotransferred to Hybond-ECL nitrocellulose (Amersham Pharmacia, Arlington, VA). For Western immunoblotting, the membranes were blocked in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with primary antibodies diluted in TBST for 2 h at room temperature or overnight at 4°C. Membranes were washed in TBST and then incubated with horseradish peroxidase-conjugated secondary antibodies (Southern Biotechnology Associates, Birmingham, AL), diluted 1:7000, for 1 h at room temperature. Immunoreactive proteins were detected using the Renaissance Western Blot Chemiluminescence reagents (New England Nuclear). For Western ligand blotting, the membranes were incubated overnight with 1.0×10^6 cpm of ^{125}I-labeled IGF-I and IGF-II after blocking with normal saline containing 1% BSA at room temperature for 2 h. The filters were washed twice with normal saline containing 0.1% Tween 20 and three times with normal saline for 15 min each, dried, and exposed to film.

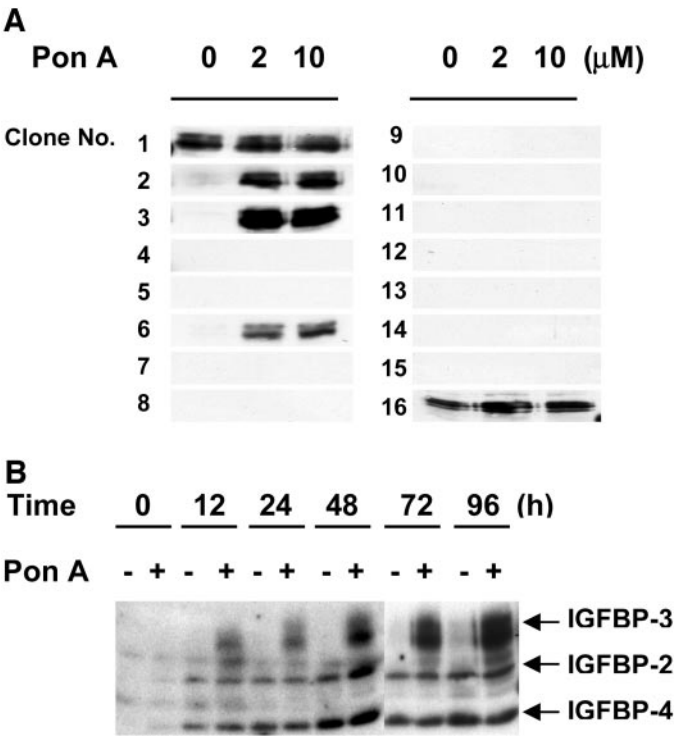

Fig. 1. Insulin-like growth factor-binding protein (IGFBP)-3 expression in stably transfected MCF-7 cells (MCF-7:IGFBP-3 cells) after treatment with ponasterone A. *A*, Western immunoblot analysis of IGFBP-3 in a panel of 16 stably transfected clones. MCF-7 cells were transfected with an expression vector containing IGFBP-3 cDNA (pIND plasmid) and a second plasmid expressing ecdysone receptor (VgEcR) and retinoid X receptor (RXR). Stably transfected cells were selected by G418 and Zeocin. Ponasterone A induces the heterodimer of RXR and VgEcR to bind the hybrid ecdysone response element in the pIND vector, resulting in the activation of IGFBP-3 transcription. Subclones 1 and 16 constitutively express IGFBP-3; subclones 2, 3, and 6 express IGFBP-3 in an inducible manner. *B*, Western ligand blotting showing that only the expression of IGFBP-3, but not that of other IGFBPs, was induced in conditioned media after treatment with ponasterone A.

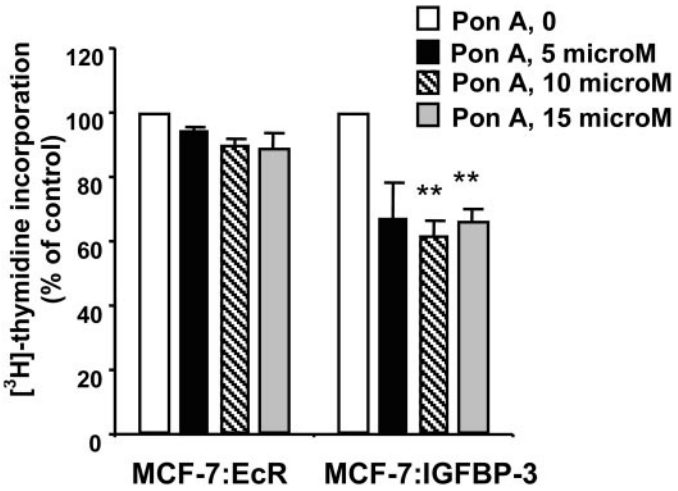

Fig. 2. Inhibitory effect of insulin-like growth factor-binding protein 3 on DNA synthesis in inducible stably transfected MCF-7 cells. Cells of MCF-7:IGFBP-3 or the parental cell line expressing only the ecdysone receptor heterodimer (EcR) were treated with ponasterone A at concentrations of 0, 5, 10, and 15 μM for 72 h in phenol red-free serum-free media. After 68 h, [^{3}H]thymidine was added, and its incorporation into DNA was determined after an additional 4 h. Each *bar* represents the mean ± SE of three independent experiments. **, $P < 0.005$, ponasterone A-treated cells *versus* cells in serum-free media on day 3.

Densitometric and Statistical Analysis. Densitometric measurement of immunoblots was performed using a Bio-Rad GS-670 imaging densitometer (Bio-Rad). All experiments were conducted at least three times. The data were analyzed with Student's *t* test, using the Microsoft Excel 98 software package.

RESULTS

Ecdysone-Inducible Expression of IGFBP-3 in the Stably Transfected MCF-7 Cells. Because recent studies suggest that posttranslational modification of IGFBP-3, such as glycosylation, phosphorylation, and proteolysis, influences the biological activity of IGFBP-3 (38–40), an ecdysone-inducible expression system was used to investigate the biological function of intracellularly expressed IGFBP-3 (41). It was first determined whether ponasterone A could induce IGFBP-3 expression in stably transfected MCF-7 cells. Among the clones picked as stable transfectants, IGFBP-3 was expressed in subclones 2, 3, and 6 in an inducible manner and was constitutively expressed in subclones 1 and 16 (Fig. 1*A*). Expression of IGFBP-3 could be detected by 12 h at concentrations of ponasterone A ranging from 2 to 10 μM without affecting cell viability. Subclone 3 was used for further investigation because physiologically relevant levels of IGFBP-3 were induced by a low concentration of ponasterone A, and noninduced levels were undetectable. The addition of ponasterone A increased the expression of IGFBP-3, but not that of the other IGFBPs, in CM (Fig. 1*B*). Quantitative analysis of IGFBP-3 concentrations in CM by an IGFBP-3 radioimmunoassay kit indicated that the maximal expression of IGFBP-3 (124 ng/ml) occurs on day 3 at a concentration of 15 μM ponasterone A.

Inhibition of DNA Synthesis by IGFBP-3. Subclone 3 MCF-7 cells were first tested for effects on cell growth by analyzing the effects of IGFBP-3 induction on DNA synthesis. The induced

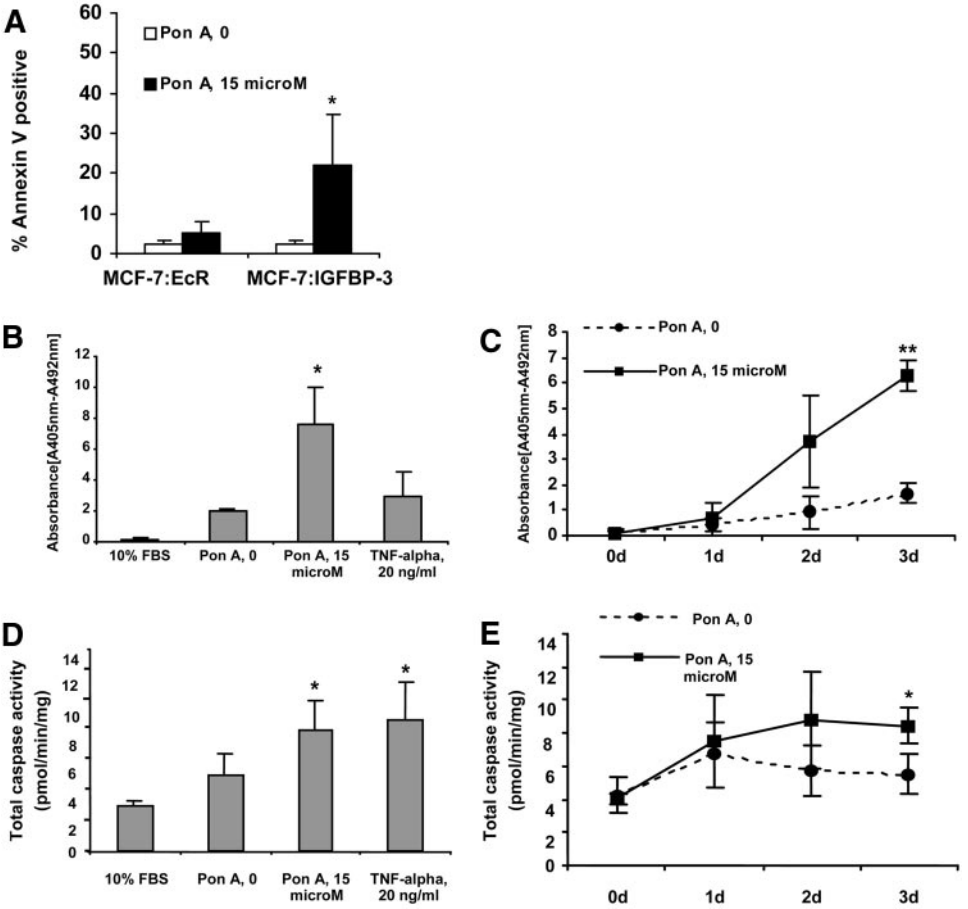

Fig. 3. Induction of apoptosis and activation of caspases by insulin-like growth factor-binding protein (IGFBP)-3 in stably transfected MCF-7 cells. *A*, MCF-7:IGFBP-3 cells or the parental cell line expressing only the ecdysone receptor heterodimer (EcR) were seeded with or without 15 μM ponasterone A in DMEM containing 10% fetal bovine serum for 72 h. Cells were incubated with annexin V, and the binding of annexin V was determined by flow cytometry. IGFBP-3-induced cells show an increase in binding of annexin V, an indicator of cells undergoing apoptosis. *, $P < 0.05$ (mean ± SE; $n = 3$). *B*, MCF-7:IGFBP-3 cells were seeded with or without ponasterone A in phenol red-free serum-free media (SFM) for 72 h. The degree of apoptosis was determined by cell death detection ELISA as described in "Materials and Methods." *, $P < 0.05$, ponasterone A-treated cells *versus* cells in SFM (mean ± SE; $n = 6$). *C*, cell death detection ELISA at the indicated time. **, $P < 0.0005$, ponasterone A-treated cells *versus* cells in SFM on day 3 (mean ± SE; $n = 6$). *D*, total caspase activity (in pmol/min/mg) was examined in MCF-7:IGFBP-3 cells with or without ponasterone A cultured in phenol red-free SFM for 72 h. *, $P < 0.05$, treated cells *versus* cells in SFM (mean ± SE; $n = 6$). *E*, total caspase activity at the indicated time. *, $P < 0.05$, ponasterone A-treated cells *versus* cells in SFM on day 3 (mean ± SE; $n = 6$).

expression of IGFBP-3 by ponasterone A resulted in an inhibition of DNA synthesis of up to 32.5–38% at concentrations of 5–15 μM ponasterone A (Fig. 2). Importantly, in the same experiments, the parental cells expressing the ecdysone receptor heterodimer (EcR cells) did not show any significant change in [^{3}H]thymidine incorporation after treatment with ponasterone A (Fig. 2).

IGFBP-3 Induces Apoptosis in MCF-7 Cells. To identify the mechanism of the inhibitory effect of IGFBP-3 on monolayer growth, we first determined whether the cellular expression of IGFBP-3 can induce apoptosis using an annexin V binding assay and a cell death detection ELISA (42). Induced expression of IGFBP-3 caused an increase in the percentage of cells undergoing apoptosis from 1.5% in the absence of ponasterone A to 21.8% in the presence of ponasterone A ($P < 0.05$ compared with cells without ponasterone A), suggesting that IGFBP-3 can induce apoptosis in this cell system (Fig. 3*A*). In the same experiments, the parental MCF-7:EcR cells did not show any significant change in the percentage of cells undergoing apoptosis after treatment with ponasterone A (Fig. 3*A*). This finding was corroborated by an independent cell death detection ELISA, in which cells cultured in DMEM supplemented with 10% FBS showed a near absence of apoptotic cells, whereas SFM-treated cells showed a basal level apoptotic index (Fig. 3*B*). In contrast, the addition of ponasterone A to the cells in SFM induced a further increase in the apoptotic index above the basal level ($P < 0.05$ compared with cells in SFM; Fig. 3*B*). The induction of apoptosis by IGFBP-3 measured using the cell death detection ELISA was greater than that induced by TNF-α, which has been demonstrated to be a potent apoptosis-inducing agent in MCF-7 cells (43). The induction of apoptosis was observed from day 2, with maximal induction on day 3 (about a 6-fold induction) after the addition of ponasterone A (Fig. 3*C*).

We next investigated whether the mode of IGFBP-3-induced apoptosis involved the activation of caspase activity specific for apoptosis. Similar to the observations obtained using the cell death detection ELISA, the induced expression of IGFBP-3 increased the level of total caspase activity ($P < 0.05$ compared with cells in SFM) to the levels seen after treatment with 20 ng/ml TNF-α (Fig. 3*D*). The increase of caspase activity was observed from day 1, with a maximal increase on day 3 (Fig. 3*E*). A slight increase of caspase activity was also observed over the incubation time in cells in SFM, which might be due to serum deprivation (Fig. 3*E*).

Effects of IGFBP-3 Are Mediated through IGF-Independent Action. We next determined whether the effects of IGFBP-3 on cell growth, induction of apoptosis, and caspase activation were IGF independent. The observed biological effects of IGFBP-3 did not result from blocking the endogenous IGF action because IGFBP-5, which binds both IGF-I and IGF-II with high affinity, had no significant effect on basal DNA synthesis in MCF-7:IGFBP-3 cells without ponasterone A when added at 150 ng/ml but blocked the stimulatory effect of 5 ng/ml IGF-I (Fig. 4*A*). Furthermore, when MCF-7 cells were transfected with GGG-IGFBP-3, an IGFBP-3 mutant unable to bind IGFs, DNA synthesis was decreased compared with the level shown in cells transfected with empty vector (Fig. 4*B*). In addition, apoptotic index and total caspase activity were increased in GGG-IGFBP-3-transfected cells (Fig. 4, *C* and *D*), which are comparable with those seen in cells overexpressing intact IGFBP-3 (Figs. 2 and 3). Taken together,

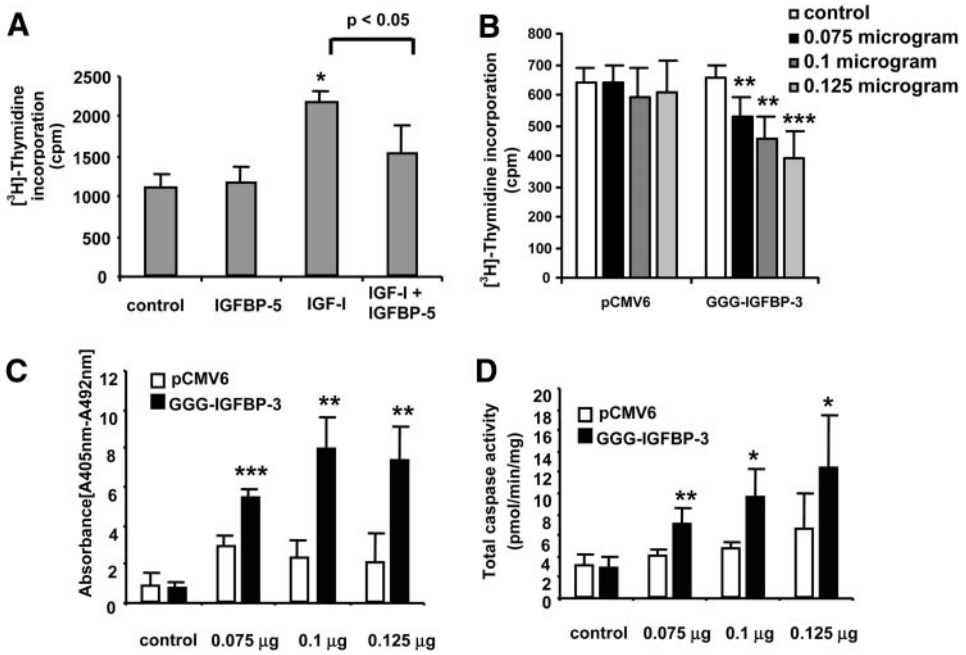

Fig. 4. Effects of insulin-like growth factor-binding protein (IGFBP)-3 in an insulin-like growth factor (IGF)-independent manner. *A*, MCF-7:IGFBP-3 cells without ponasterone A were treated with human IGFBP-5 (150 ng/ml), IGF-I (5 ng/ml), or both in phenol red-free serum-free media (SFM) for 24 h before [^{3}H]thymidine incorporation assay. *, $P < 0.05$, control *versus* IGF-I-treated cells. *B*, MCF-7 cells were seeded on 24-well culture plates in DMEM supplemented with 10% fetal bovine serum. At 60–70% confluence, cells were transfected with pCMV6 vector or pCMV6/GGG-IGFBP-3. Twenty-four h after transfection, the media were replaced with phenol red-free SFM. After 68 h, [^{3}H]thymidine was added, and its incorporation into DNA was determined after an additional 4 h. **, $P < 0.005$; ***, $P < 0.0005$ (control *versus* GGG-IGFBP-3-transfected cells). *C*, the degree of apoptosis was determined by cell death detection ELISA in cells incubated with phenol red-free SFM for 72 h after transfection. **, $P < 0.005$; ***, $P < 0.0005$ (control *versus* GGG-IGFBP-3-transfected cells). *D*, total caspase activity was examined in cells incubated with phenol red-free SFM for 72 h after transfection. *, $P < 0.05$; **, $P < 0.005$ (control *versus* GGG-IGFBP-3-transfected cells). Each *bar* represents the mean ± SE of three independent experiments.

these data confirm that effects on cell growth and induction of apoptosis are IGFBP-3 specific and IGF-IGF receptor independent.

Caspases Involved in a Death Receptor-Mediated Pathway Are Activated in IGFBP-3-Induced Apoptosis. To determine which caspases are activated by IGFBP-3, we measured the kinetics of IGFBP-3-induced changes in enzyme activities using substrates that display specificity for each caspase *in vitro*: Ac-IETD-AMC (caspase-8), Ac-LEHD-AMC (caspase-9), Ac-DEVD-AMC (caspase-3/-7), or a mixture of Ac-DEVD-AMC and Ac-LEHD-AMC (total caspase), respectively. It was observed that the induced expression of IGFBP-3 significantly increased total caspase, IETDase, and DEVDase activity (Fig. 5*A*, *, $P < 0.05$ compared with cells with SFM) but not LEHDase activity. Because MCF-7 cells do not express a functional caspase-3 due to a deletion within exon 3 of the *CASP3* gene (44), the DEVDase activity may come from the activity of caspase-7 or -2 in our cell system. In contrast, increased IETDase activity may come from the activation of caspase-8, -10, or -6 on the basis of Ac-IETD-AMC specificity (32). To identify the caspases involved in IGFBP-3-induced apoptosis, the activation of procaspase-8, -10, -6, -9, -7, and -2 was examined in cells treated with ponasterone A by Western immunoblotting (Fig. 5*B*). As expected from the caspase activity studies, we found a significant decrease in the level of procaspase-8, and -7, but not procaspase-10, -6, -9, and -2, after the addition of ponasterone A. Maximal decrease of procaspase-8 and -7, accompanied by an increase of cleaved PARP, was observed on day 3 after the addition of ponasterone A, suggesting that IGFBP-3 activates caspase-8 and -7, although the cleaved caspases were not detected in our cell system.

IGFBP-3-Induced Apoptosis Is Mediated by Caspase-Dependent Pathways. Previous studies have demonstrated that exposure of MCF-7cells to TNF-α (25–50 ng/ml) leads to apoptotic cell death (30–60%) within 24 h (43). In the present study, cells treated with 20 ng/ml TNF-α or 15 μM ponasterone A both showed a marked decrease in proliferation (Fig. 6*A*) and an increased apoptotic index (Fig. 6*B*), as well as elevated caspase activity (Fig. 6*C*) on day 2 after treatment. Furthermore, TNF-α and IGFBP-3-induced cell death was accompanied by an increase in the level of cleaved PARP (Fig. 6*D*). These changes induced by TNF-α or IGFBP-3 were completely prevented by cotreatment with the broad-spectrum caspase inhibitor Z-VAD-FMK (Fig. 6, *A*–*D*), suggesting that both TNF-α- and IGFBP-3-induced apoptosis are mediated by caspase-dependent pathways. At the same time, the caspase-8 inhibitor Z-IETD-FMK also completely abolished the changes in cell proliferation, apoptotic index, caspase activity, and cleaved PARP expression by TNF-α or IGFBP-3 (Fig. 6, *A*–*D*), confirming that caspase-8 is a key initiator caspase involved in the induction of apoptosis by IGFBP-3.

IGFBP-3 Does Not Affect the Release of Cytochrome *c* into the Cytosol in MCF-7 Cells. Mitochondrial release of cytochrome *c* into the cytosol during apoptosis is thought to be caspase independent, even though in some cases of death receptor-mediated apoptosis, caspase-8 or -10 may act on mitochondria to facilitate cytochrome *c* release, thereby further amplifying the apoptotic signal (45). To determine whether IGFBP-3 triggers cytochrome *c* release, we examined the amount of cytochrome *c* in the cytosolic and mitochondrial fractions of cells after treatment with ponasterone A. Because TNF-α has been reported to elicit mitochondrial cytochrome *c* release and Bid cleavage in MCF-7 cells (43), cells were treated with 20 ng/ml TNF-α for 48 h as a positive control. There was minimal cytochrome *c* in the cytosolic fraction in SFM-cultured and ponasterone A-treated cells, whereas detectable cytochrome *c* was increased in TNF-α-treated cells (Fig. 7, *top panel*). Simultaneously, the amount of cytochrome *c* in the mitochondrial fraction was decreased in TNF-α-treated cells, but not in the SFM-cultured and ponasterone A-treated cells (Fig. 7, *top panel*). Bid is normally cleaved by a caspase-8-dependent process, and cleaved Bid elicits mitochondrial cytochrome *c* release. The amount of intact Bid in the cytosolic fraction was decreased in TNF-α-treated cells, whereas no change was observed in ponasterone A-treated cells or SFM-cultured cells (Fig. 7, *middle panel*). These results suggest that IGFBP-3 does not affect the release of cytochrome *c* from mitochondria.

DISCUSSION

IGFBP-3, which is the most abundant IGFBP in human serum, acts not only as a carrier of IGFs, thereby prolonging their half-

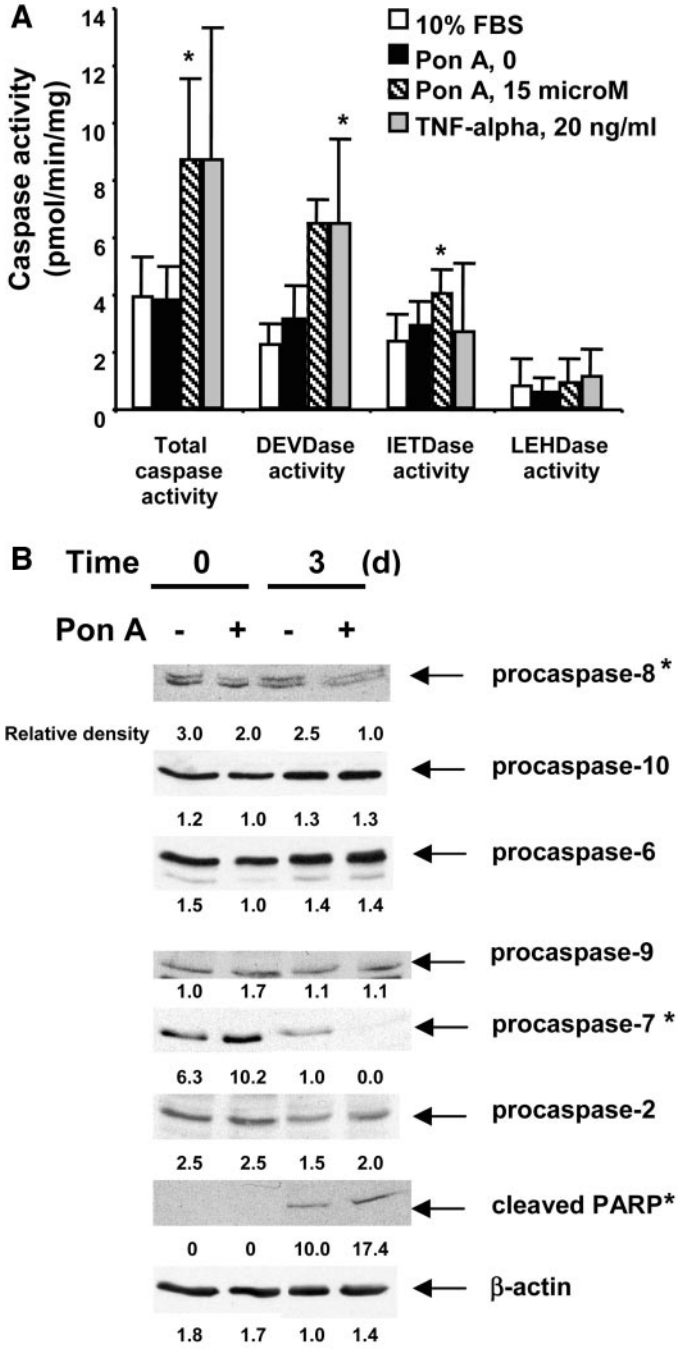

Fig. 5. Activation of caspases involved in death receptor-mediated pathway in insulin-like growth factor-binding protein 3-induced apoptosis in MCF-7 cells. *A*, lysates of cells incubated with or without ponasterone A or in 10% fetal bovine serum or 20 ng/ml tumor necrosis factor α for 72 h were assayed for total caspase (DEVDase and LEHDase), DEVDase (caspase-3-like), IETDase (caspase-8), and LEHDase (caspase-9) activities (mean $\pm$ SE; $n = 6$). $*$, $P < 0.05$, treated cells *versus* cells in serum-free media. *B*, Western immunoblot analysis of caspase-8, -10, -6, -9, -7, and -2 and cleaved PARP in MCF-7:IGFBP-3 cells with or without ponasterone A at the indicated time. Proteins showing a significant change after addition of ponasterone A are indicated by an *asterisk*.

lives, but also functions as a modulator of the IGF activity by regulating the availability of free IGFs for their interaction with IGF receptors (1–2). Recently, there has been a growing accumulation of data demonstrating that IGFBP-3 may have its own biological actions, inhibiting cell proliferation in an IGF-independent manner (6–19). Conceptually, IGFBP-3 can exert its actions on target cells in several ways: (*a*) inducing apoptosis; (*b*) regulating the cell cycle; and (*c*) possible cross-talk with major signal transduction pathways. Mechanisms for inducing apoptosis, in part, have been demonstrated by studies showing that IGFBP-3 increases the ratio of proapoptotic (Bax and Bad) to antiapoptotic (Bcl-2 and Bcl-X_L) proteins in apoptotic breast cancer cells (24), and caspase-3 activity and IGFBP-3 levels in CM are increased in paclitaxel-treated Hs578T breast cancer cells (25). As a regulator of the cell cycle, IGFBP-3 has been shown to regulate the induction of the cyclin-dependent kinase inhibitory protein p21/WAF/CIP1 by vitamin D analogs in prostate cancer cells (46). In addition, an active Ras-mitogen-activated protein kinase pathway has been shown to block IGFBP-3-induced cell growth inhibition, whereas inhibition of the mitogen-activated protein kinase pathways restored the biological function of IGFBP-3 (47). Although these early investigations have revealed, in part, suggestive mechanisms for IGFBP-3 action, mechanisms of action for IGFBP-3 remain largely undetermined.

In the present investigation, we have begun to address specific questions regarding the intracellular mechanisms involved in IGFBP-3-induced apoptosis in MCF-7 breast cancer cells. To determine these mechanisms, we used MCF-7 cells expressing cellular IGFBP-3 in an inducible manner. Because MCF-7 cells do not express detectable IGF-I (48–50), and experiments in this study were performed in phenol red-free SFM, our results suggest that the apoptosis-inducing effect of IGFBP-3 in MCF-7 cells is independent of the IGF-IGF receptor axis, although the model system does not rule out the effects of residual IGFs. Nevertheless, use of a IGFBP-3 mutant and other IGFBPs confirms that IGFBP-3-induced cell growth inhibition is specific for IGFBP-3 and IGF-independent: (*a*) MCF-7:IGFBP-3 cells used in this study are not inhibited by IGFBP-5, which binds both IGF-I and -II with high affinity; and (*b*) more definitively, MCF-7 cells transfected with the IGFBP-3 mutant, which is unable to bind IGFs, show inhibition of DNA synthesis, induction of apoptosis, and activation of caspase.

Here we demonstrate for the first time that IGFBP-3 induces apoptosis in MCF-7 cells by activating multiple caspases involved in a death receptor-mediated pathway, such as caspase-8 and -7, but not via the mitochondria-mediated pathway. Furthermore, these findings do not reflect clonal variation of the MCF-7:IGFBP-3 cells used in this study because the expression of IGFBP-3 by adenoviral transduction results in the same biological effects in MCF-7 cells (data not shown). Despite the lack of functional caspase-3 due to a deletion in the *CASP3* gene (44), MCF-7 cells underwent apoptosis, suggesting that other executioner caspases, such as caspase-7 or -2, may be involved. We demonstrate that IGFBP-3 activates caspase-7 but not caspase-2, as shown by Western immunoblotting, explaining the elevated caspase-3-like (DEVD-ase) activity, which is consistent with previous studies showing that caspase-7 was processed in doxorubicin-induced apoptosis of MCF-7 cells (51), but caspase-2 was not processed after TNF-α or staurosporine treatment (52). Caspase-8 can activate caspase-3 and -7 directly and/or through triggering the activation of caspase-9 by the release of cytochrome *c* from mitochondria (26–29). To assess the relative importance of caspase-8 in the cytotoxic signaling of IGFBP-3, we examined the effect of Z-IETD-FMK (caspase-8 inhibitor) during IGFBP-3-induced apoptosis. The caspase-8 inhibitor significantly blocked the actions of IGFBP-3 to increase total caspase activity, to induce apoptosis as measured by the cell death detection ELISA assays, to cleave PARP, and to suppress cell proliferation. Thus, on the basis of this caspase activity assessment and the studies using the caspase-8 inhibitor, IGFBP-3-induced apoptosis in MCF-7 cells involves the caspase-8-mediated activation of executioner caspases such as

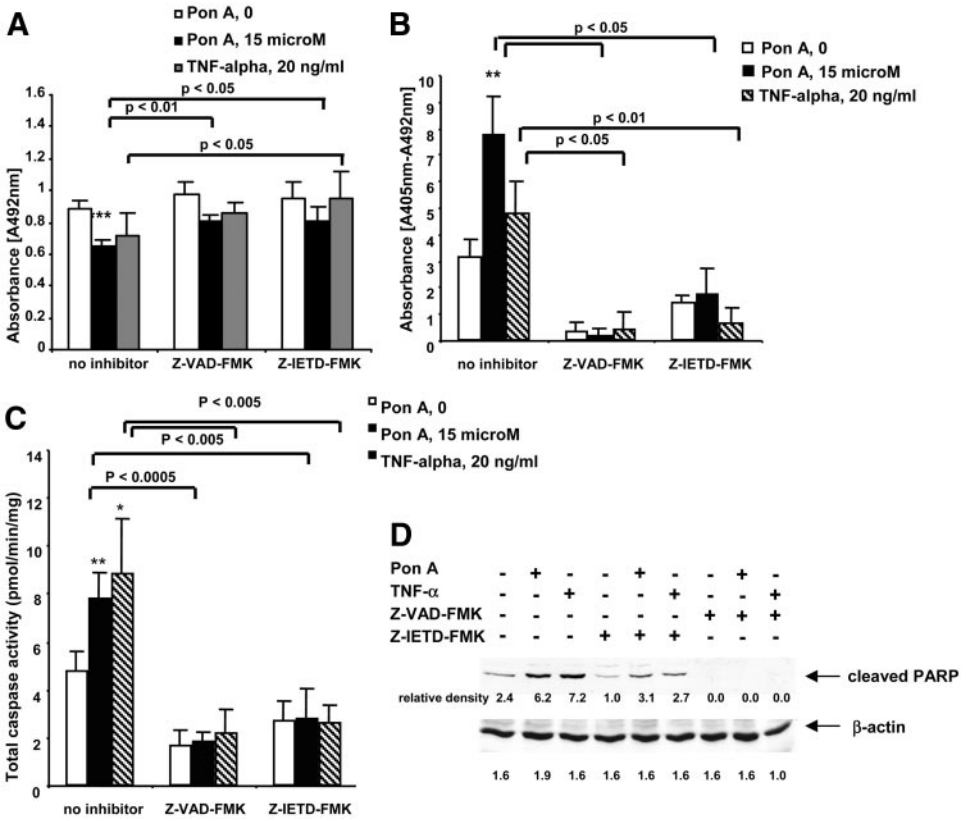

Fig. 6. Activation of caspases by insulin-like growth factor-binding protein 3 in a caspase-dependent manner. MCF-7:IGFBP-3 cells were incubated with 15 μM ponasterone A or 20 ng/ml tumor necrosis factor α in phenol red-free serum-free media $\pm$ 25 μM Z-VAD-fluoromethylketone or 20 μM Z-IETD-fluoromethylketone for 72 h. *A*, cell proliferation assay by CellTiter 96 assay. *B*, apoptotic index by cell death detection ELISA. *C*, total caspase activity. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ (treated cells *versus* cells in serum-free media; mean $\pm$ SE; $n = 6$). *D*, Western immunoblot analysis of cleaved PARP.

caspase-7, but not the activation of caspase-9. At the same time, the broad-spectrum caspase inhibitor Z-VAD-FMK also abolished the negative effects on cell proliferation, induction of apoptosis, caspase activity, and levels of cleaved PARP by IGFBP-3, suggesting that the antiproliferative effect of IGFBP-3 results mainly from the induction of apoptosis. During the preparation of this paper, Butt *et al.* (53) also reported that IGFBP-3-mediated apoptosis in T47D human breast cancer cells was significantly blocked by treatment with Z-VAD-FMK, further supporting our findings.

Cytochrome *c* release from mitochondria during apoptosis can be achieved through two different mechanisms. Direct stimuli such as genotoxic agents or UV light can release cytochrome *c* in a caspase-independent manner, whereas activated caspase-8 or -10 may also facilitate cytochrome *c* release from mitochondria,

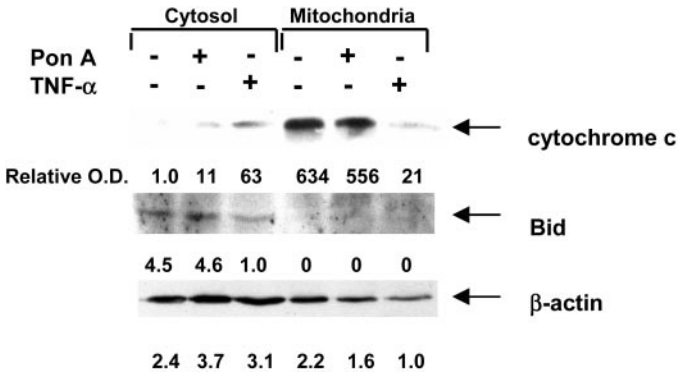

Fig. 7. Subcellular distribution of cytochrome *c* after induction of insulin-like growth factor-binding protein 3 in MCF-7 cells. MCF-7:IGFBP-3 cells were incubated with 15 μM ponasterone A or 20 ng/ml tumor necrosis factor α in phenol red-free serum-free media for 48 h. Cytosolic and mitochondrial fractions prepared as described in "Materials and Methods" were immunoblotted with monoclonal antibody to cytochrome *c* and Bid. The results are representative of at least three independent experiments.

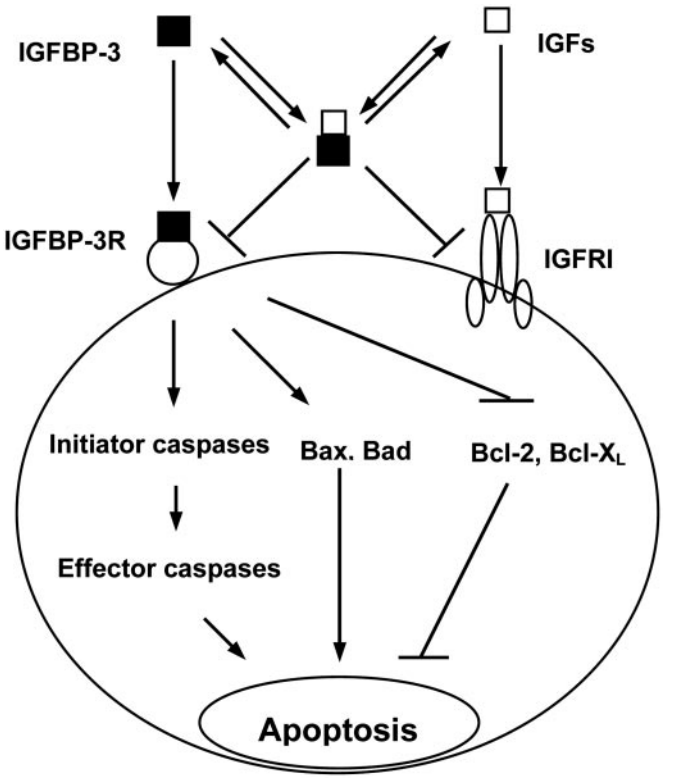

Fig. 8. Schematic representation of insulin-like growth factor-binding protein (IGFBP)-3-induced apoptosis. IGFBP-3 may inhibit cell growth by induction of apoptosis either indirectly, by sequestrating insulin-like growth factors away from the insulin-like growth factor receptor, or directly via putative IGFBP-3 receptor and/or translocation to the nucleus. IGFBP-3 increases the ratio of proapoptotic (Bax and Bad) to antiapoptotic (Bcl-2 and Bcl-X_L) members of the Bcl-2 family (24). Our study demonstrates the existence of an apoptotic cascade, which utilizes caspases involved in the death receptor-mediated pathway such as caspase-8 and -7.

thereby further amplifying the apoptotic signal (48). In our study, the induced expression of IGFBP-3 did not affect the redistribution of cytochrome *c*, suggesting that IGFBP-3-induced apoptosis does not result from the disruption of mitochondrial function and caspase-9 activation, which is consistent with previous studies on cisplatin- or somatostatin-induced apoptosis in MCF-7 cells (54, 55). Minimal detection of cytochrome *c* in the cytosol of both control cells and ponasterone A-treated cells may result from serum deprivation as observed previously (56).

In summary, these findings help define the temporal sequence of events that links the initiator and effector caspases in IGFBP-3-induced apoptosis in MCF-7 cells. Fig. 8 depicts the proposed mechanism for IGFBP-3-induced apoptosis in MCF-7 cells, based on the results from the present study and other previous studies (24). The present study supports the existence of an IGFBP-3-regulated apoptotic cascade, which utilizes caspases involved in death receptor-mediated pathways, such as caspase-8 and caspase-7, suggesting that IGFBP-3 functions as a negative regulator of breast cancer cell growth, independent of the IGF axis. These events occur on the cell surface as demonstrated previously in the prostate cancer cell system (20). It is tempting to speculate that IGFBP-3-induced apoptosis may be initiated by interaction of IGFBP-3 with the cognate receptor(s), which has yet to be identified, thereby subsequently activating initiator caspase-8 and downstream effector caspases.

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