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Overexpression of BCL-X_L Underlies the Molecular Basis for Resistance to Staurosporine-induced Apoptosis in PC-3 Cells[1]

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ABSTRACT

We have reported previously that among human prostate cancer cell lines LNCaP but not PC-3 cells undergo apoptosis after treatment with the protein kinase inhibitor staurosporine (STS). We have now further investigated this model to uncover the molecular mechanism causing resistance to STS-induced apoptosis in PC-3 cells. S-100 lysates of both cell lines showed biochemical changes typical of apoptosis after the addition of cytochrome *c* and dATP, suggesting that the postmitochondrial phase of apoptosis was intact. Upon addition of STS, the proapoptotic molecules Bax and Bad became predominantly mitochondrial in both cell lines. This, in turn, was followed by loss of mitochondrial transmembrane potential, translocation of cytochrome *c* to the cytosol, activation of caspase-9, -3, and -7, and cleavage of the apoptotic targets, DNA fragmentation factor and poly(ADP-ribose) polymerase, in LNCaP but not in PC-3 cells. Components of the mitochondrial permeability transition pore, adenine nucleotide transporter and voltage-dependent anion channel, were normally expressed in the correct subcellular fraction of both cell lines. Overexpression of the proapoptotic proteins Bax and Bad, fused to a green fluorescent protein but not of green fluorescent protein alone, induced apoptosis in >80% of PC-3 cells. These experiments suggested that a factor protecting the mitochondria of PC-3 cells mediates resistance to STS-induced apoptosis. A wide search among the antiapoptotic Bcl-2 family members was performed, and Bcl-X_L was found to be overexpresseÃd in PC-3 cells. Experiments down-regulating Bcl-X_L expression by using the tyrosine kinase inhibitor genistein, sodium butyrate, or an antisense Bcl-X_L oligonucleotide restored sensitivity to apoptosis in PC-3 cells. Thus, Bcl-X_L overexpression is one of the mediators of resistance to STS-induced apoptosis in the prostate cancer cell line PC-3.

INTRODUCTION

Three major apoptotic pathways originating from three separate subcellular compartments have been identified. The receptor-mediated pathway involves the interaction of plasma membrane death receptors with their ligands (1). The mitochondrial pathway involves the functional incapacitation of the mitochondria by proapoptotic Bcl-2 family members (2). Finally, the endoplasmic reticulum pathway (3) is elicited by the application of various forms of stress that result in activation of caspase-12. Although each pathway is initially centered around unique events, the final phase of apoptosis is thought to be common and consists in the activation of the executioner caspases and in their dismantling of substrates critical for cell survival (4). The mitochondrial pathway is thought to be the main pathway activated after treatments with chemotherapeutic agents such as STS[5] and sodium phenyl acetate (5), the removal of critical growth factors, such as interleukin 3 in FL5.12 cells (6), or UV irradiation (7). Three major phases have been described for the mitochondrial pathway. In the premitochondrial phase, there is disruption of the survival pathways that inactivate proapoptotic molecules (8–13) or facilitate the formation of new antiapoptotic factors (14, 15). In the mitochondrial phase, proapoptotic members of the Bcl-2 family of factors become bound to the mitochondria (6, 16–18), cause loss of the mitochondrial transmembrane potential (19), and release to the cytosol of apoptotic molecules such as cytochrome *c* (20), the second mitochondria-derived activator of caspase (DIABLO; Ref. 21, 22), and the apoptosis-inducing factor (23). Finally, in the postmitochondrial phase there is the assembling of the apoptosome (24), activation of the caspase pathway (4), disintegration of cellular contents, and subsequent absorption by neighboring cells.

A major shortcoming of current cancer chemotherapy is that many types of cancers acquire resistance to the apoptotic effect of various cytotoxic agents and become progressively incurable. Prostate cancer, which is resistant to hormone withdrawal treatment, is a particularly good example. Most prostate cancer cells respond initially to androgen ablation treatment (orchiectomy, diethylstilbestrol, luteinizing-hormone releasing-hormone analogues, and androgen receptor antagonists given alone or in combination) by undergoing apoptosis (25). However, after 12 to 18 months most prostate cancer cells become androgen-independent and apoptosis-resistant (26), and this eventually leads to overwhelming disease and death.

Unraveling the mechanisms protecting prostate cancer cells from undergoing apoptosis after androgen ablation would facilitate comprehension of the basic cellular mechanisms leading to prostate cancer cell survival after this form of treatment. It would also facilitate identification of new therapeutic targets and development of new treatments. Unfortunately, reproducible models of prostate cancer cell lines developing progressive resistance to androgen ablation-induced apoptosis are not available. Nevertheless, differences in sensitivity to apoptotic agents other than androgen-ablation have been identified among various prostate cancer cell lines. For instance, among the various prostate cancer cell lines, we and others have observed that PC-3 cells are more resistant to apoptosis than LNCaP cells (27–29). Our group has characterized previously in detail how PC-3 and LNCaP cells respond to the apoptotic agent STS. In the presence of this protein kinase inhibitor, LNCaP cells undergo massive apoptosis after as little as 3 to 6 h because of the activation of the mitochondrial pathway (5, 27, 30). In contrast, STS does not activate the mitochondrial pathway in PC-3 cells, which are still largely viable after as long as 72 h of treatment (27). Hypothesizing that elucidating the mechanism causing STS resistance in PC-3 cells would shed light on the

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[5] The abbreviations used are: STS, staurosporine; VDAC, voltage-dependent anion channel; ANT, adenine nucleotide transporter; DFF, DNA fragmentation factor; PARP, poly(ADP-ribose) polymerase; RT-PCR, reverse transcription-PCR; GFP, green fluorescent protein; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

major survival pathways active in androgen-independent prostate cancer cells and in other cancer cells resistant to chemotherapy, we performed a careful dissection of the events occurring (and not occurring) in LNCaP and PC-3 cells after STS treatment. Our analysis finds that overexpression of the antiapoptotic factor Bcl-X_L is one of the mediators of resistance to STS in PC-3 cells in that it protects the mitochondria from undergoing functional incapacitation.

MATERIALS AND METHODS

Materials. Fetal bovine serum and tissue culture media were from Life Technologies, Inc. (Frederick, MD). STS was from Alexis Corporation (San Diego, CA). Genistein was from Research Biochemicals International (Natick, MA). Sodium butyrate was from Sigma Chemical Co. (St. Louis, MO). Antibodies for caspase-3, caspase-7, Bcl-2, and Bcl-X were from Transduction Laboratories (Lexington, KY). The anti-cytochrome *c*, -Bax, and -caspase-9 antibodies were from PharMingen (San Diego, CA). The anti-Bid and DFF antibodies were gifts of Dr. Wang (University of Texas Southwestern, Dallas, TX). The anti-Bad antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-VDAC and ANT antibodies were gifts of Drs. W. Craigen (Baylor College of Medicine, Houston, TX) and H. Schmid (Hormel Institute). The anti-β-actin antibody was from Sigma Chemical Co., and the anti-tubulin was from Chemicon International, Inc. (Temecula, CA). The anti-PARP antibody was from Biomol (Plymouth Meeting, PA). The *In Situ* Cell Death Detection Kit and the Cellular DNA Fragmentation ELISA kit were from Boehringer Mannheim (Indianapolis, IN). The fluorogenic substrate Z-DEVD-AFC was from Enzyme Systems (Dublin, CA). JC-1 was from Molecular Probes (Eugene, OR). RT-PCR was done using the RNA amplification kit from Roche (Branchburg, NJ). The enhanced chemiluminescence detection kit was from Amersham Corp. (Arlington Heights, IL). TUNEL positive cells were scored using a fluorescence microscope (Olympus IX70; Olympus America, Melville, NY). Images were recorded with a digital camera SPOT (Diagnostic Instruments, Sterling Heights, MI). Confocal analysis was performed on an Olympus IMT2 microscope equipped with a Bio-Rad MRC 1024 (Bio-Rad Laboratories, Hercules, CA) scanning apparatus.

Plasmids. Plasmid GFP-Bax was a gift of R. Youle (31). Plasmid GFP-Bad was constructed using a Bad cDNA obtained by PCR from a LNCaP cDNA library. The following primers containing the restriction sites *Xho*I and *Xba*I were used: Bad-S, CAAGATCTCATCTTGTCCTCACAGCCCAGAGC; Bad-AS, CATCTAGATCACTGGGAGGGGGCGGAGCTTCCCCT. The product of the amplification was cut with the two restriction endonucleases *Xho*I and *Xba*I and subcloned in a pEGFP-C1 plasmid (Clontech Laboratories, Inc., Palo Alto, CA) cut with the same enzymes. Sequence analysis was performed to ensure that the two constructs were in-frame and that no artifacts were added to the Bad sequence by the amplification process. A VDAC cDNA inserted in the expression plasmid pRc/RSV (Invitrogen, San Diego, CA) was a gift of M. Forte (Vollum Institute, Portland, OR).

Cell Lines and Experimental Design. LNCaP (32) and PC-3 (33) cells have been described previously (5, 27). Two days before the subcellular localization experiments, 1×10^5 cells were seeded in a 6-well plate. At the time point 0, cells were treated with 4 μM STS from a 4 mM stock dissolved in DMSO. Adherent and floating cells were recovered 6 h (LNCaP) or 24 h (PC-3) after STS stimulation.

Genistein was given to PC-3 cells at a concentration of 300 μM (from a stock of 300 mM dissolved in DMSO) for 24 h. Vehicle-treated controls were given the same amount of DMSO. Adherent and floating cells were harvested at 24 h after genistein and analyzed for the presence of various markers of apoptosis (see below).

In other experiments, PC-3 cells were treated with sodium butyrate. Cells received 2 mM sodium butyrate (dissolved in water) for 24 or 48 h. Parallel dishes were treated with the same amount of sodium butyrate plus 4 μM STS during the final 24 h of the experiment. Control dishes were treated with vehicle or STS alone. Cells were then collected and studied for various markers of apoptosis (see below).

Cell-free Assay of Apoptosis. As a diagnostic test for the postmitochondrial phase of apoptosis, we used the cell-free assay described by Liu *et al.* (20). The cell line of interest was harvested and washed twice at 4°C in PBS by centrifugation at 1500 rpm. The resulting pellet was resuspended in one volume of buffer A [HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitors; Sigma Chemical Co.] and incubated at 4°C for 20 min. Cells were then disrupted through passage in a 26-gauge needle 15 times, spun at 1,000 $\times$ *g* at 4°C for 10 min. This supernatant was spun at 100,000 $\times$ *g* for 30 min at 4°C, used for protein determination, aliquoted, and stored at -80°C. Aliquots of 50 μg of protein were incubated for 30 min at 30°C alone, in the presence of dATP (1 mM), or in presence of both dATP (1 mM) and cytochrome *c* (10 μM). Aliquots containing 10 μg of protein were then sized in a polyacrylamide gel, and Western analysis for caspase-3, caspase-7, and DFF was performed.

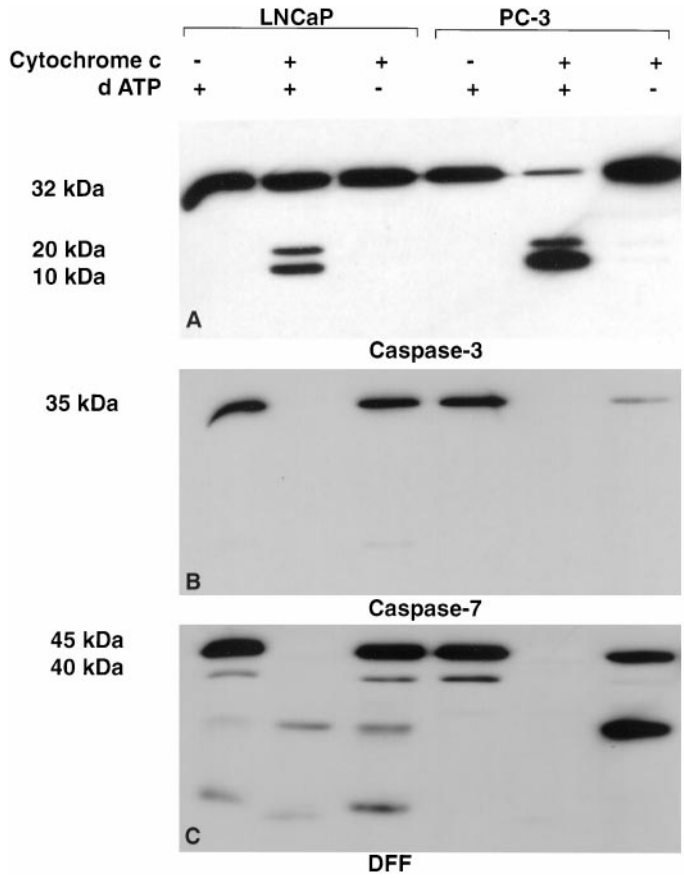

Fig. 1. Addition of cytochrome *c* and dATP is followed by three well-characterized biochemical markers of apoptosis (*i.e.*, cleavage of procaspase-3, -7, and DFF) in both LNCaP and PC-3 cells S-100 extracts. S-100 extracts (10 μg) were sized and analyzed for procaspase-3, -7, and DFF cleavage by Western analysis. Note that procaspase-3 activation (*A*) is followed by appearance of the two active subunits. Activation of procaspase-7 (*B*) is followed by disappearance of the zymogen form. Cleavage of DFF (*C*) is followed by the complete disappearance of the M_r 45,000–40,000 doublet of the molecule. Incomplete cleavage of procaspase-7 (*B*) and DFF (*C*) is also noted in PC-3 cells receiving cytochrome *c* alone. A representative experiment of two is shown.

Stable and Transient Transfections. Stable and transient transfections were performed using the LipofectAMINE-plus kit (Life Technologies, Inc.) according to the instructions of the manufacturer, unless stated otherwise. G418 (Life Technologies, Inc.) was used at a final concentration of 400 μg/ml to obtain stable transfectants when necessary.

Western Analysis and Subcellular Fractionation. Western analysis was performed as described previously (5, 27, 30). In each experiment, the same number of μg of cell lysate was loaded, as specified in each case. When precise quantitation was required, densitometric analysis was performed to correct expression of the protein of interest with that of β-actin or tubulin, which were immunodetected in the same sample. Densitometry was done by importing images to a Power Macintosh G3 personal computer using the Chemi Doc Documentation System and the Quantity One quantitation software (both from Bio-Rad, Hercules, CA). Arbitrary densitometric units of the protein of interest were then corrected for the densitometric units of β-actin or tubulin.

Subcellular fractionation was performed using serial centrifugation steps as

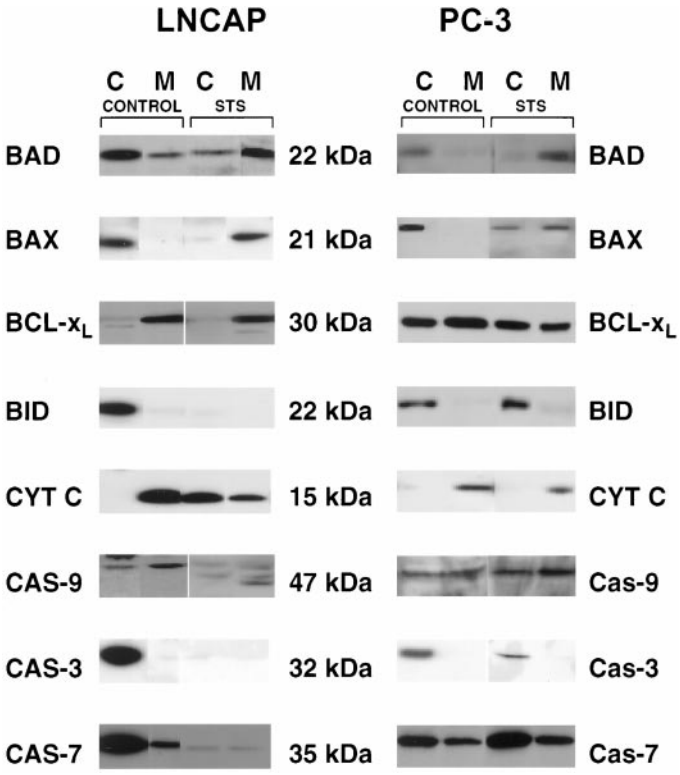

Fig. 2. Intracellular trafficking of apoptotic molecules in LNCaP and PC-3 cells before and after treatment with 4 μM STS (6 and 24 h, respectively). Cell lysates obtained from each subcellular fraction (10 μg for caspase-3, caspase-7, Bcl-X_L, Bax, and Bad; 4 μg for cytochrome *c*; and 20 μg for caspase-9 and Bid) were sized and processed as described in "Materials and Methods." A representative experiment of three is shown.

described by Gross *et al.* (34, 35) with some modifications. Briefly, LNCaP and PC-3 cells were washed twice in ice-cold PBS, resuspended in five volumes of extraction buffer [containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM MgCl2, 1 mM DTT, and protease inhibitors; Sigma Chemical Co.; added at 1:100 dilution], and kept on ice for 15 min. Cells were then spun at 400 $\times$ *g* for 10 min at 4°C to separate out nuclei and unbroken cells. This supernatant was centrifuged at 10,000 $\times$ *g* for 10 min at 4°C to collect the heavy membrane, mitochondrial-enriched pellet. The new supernatant was then spun at 100,000 $\times$ *g* for 30 min at 4°C to separate the light membrane ER-enriched pellet (not used in these experiments) from the supernatant (containing the cytosol). Pilot experiments demonstrated the ability of this technique to yield subcellular fractions enriched with mitochondria or cytosol. For instance, cytochrome *c* was recovered uniquely from the mitochondrial fraction in cells not undergoing apoptosis and from both the cytosolic and mitochondrial fractions when cells were undergoing apoptosis. In contrast, the proteins VDAC and ANT (located on the outer and inner mitochondrial membrane, respectively) were recovered uniquely from the mitochondrial fraction, regardless of whether the cells were undergoing apoptosis or not.

Immunoprecipitation. Immunoprecipitation was performed according to standard procedures (36). Cells were harvested under nondenaturing conditions and washed in ice-cold PBS. The pellet was resuspended in 500 μl of lysis buffer (PBS + 1% NP40) and 20 μl of protease inhibitor cocktail and disrupted by sonication. Protein A-Sepharose beads (10 μl; Sigma Chemical Co.) were added, and the resulting suspension was gently rocked for 10 min at 4°C and microfuged for 10 min at 4°C. Protein concentration was measured in the resulting supernatant. Supernatant (200 μl; adjusted to contain 200 μg of proteins) was taken, and the antibody of interest (anti-VDAC) was added at the ratio of 1 μg:100 μg/proteins. After overnight rocking at 4°C, 10 μl of protein A beads were added, and gentle rocking continued for an additional 4 h. The solution was then microfuged for 3 min at 4°C, and the resulting pellet was washed 2$\times$ with PBS and diluted in 40 μl of lysis buffer and 20 μl of loading buffer [62.5 Tris (pH 6.8), 10% glycerol, 2% SDS, 0.003% bromphenol blue, and 5% β-mercaptoethanol]. Twenty-μl aliquots were sized by electrophoresis and transferred to nitrocellulose. Filters were incubated with anti-Bax or -Bad antibodies, and immunoreactive bands were visualized using the enhanced chemiluminescence detection system.

Confocal Microscopy. Confocal microscopy of the mitochondrial transmembrane potential was performed using the fluorescent dye JC-1 (Molecular Probes) as reported previously (5, 27). In the presence of a negative mitochondrial transmembrane potential, this dye dimerizes and produces a red fluorescence. In contrast, when the transmembrane potential is obliterated, JC-1 produces a green fluorescence.

Immunoflurescence of Bcl-X_L and Bax was performed using PC-3 and LNCaP cells grown on coverslips. After 48 h, cells were washed, fixed with paraformaldehyde 3.7% in PBS for 10 min at room temperature, and permeabilized with 0.1% Triton X-100 for 5 min. Cells were then incubated with anti-Bax (mouse monoclonal; Santa Cruz Biotechnology) and anti-Bcl-x_L (goat polyclonal; Santa Cruz Biotechnology) antibodies for 60 min at room temperature. After extensive washes in PBS, cells were stained with 568 Alexa Fluor-conjugated antigoat antibody and 488 Alexa-conjugated antimouse antibody (Molecular Probes). Nuclei were counterstained with TO-PRO 3 (Molecular Probes). Cells were examined using an Olympus IMT-2 microscope equipped with an MRC-1024 laserscan confocal system (Bio-Rad) using the 488-nm, 568-nm, and 647-nm excitation lines from an Argo-krypton laser. Green (Bax), red (Bcl-x_L), and ultra-red emissions were collected and processed with the Lasersharp 1024 software (Bio-Rad).

Molecular Analysis of Bax and Bad in PC-3 Cells. Each individual coding exon of Bax was amplified from genomic DNA extracted from PC-3 cells using sets of primers derived from the surrounding introns, as published by Chou *et al.* (37). The resulting bands were sequenced directly using an automated sequencer (Perkin-Elmer Sequencer 310).

A full-length Bad cDNA was obtained from PC-3 cells by RT-PCR of total RNA, using random examers for the reverse transcription, and the primers Bad-S and Bad-AS were used for the amplification of Bad from LNCaP cells (see above). RT-PCR conditions were as suggested by the manufacturer of the Gene Amp kit (Roche, Brandburg, NJ.).

Identification of Apoptotic Cells after Transient Transfection of PC-3 Cells with GFP-Bad and GFP-Bax. PC-3 cells growing on coverslips were transiently transfected with GFP, GFP-Bad, and GFP-Bax using the calcium precipitation kit from 5 prime-3 prime, Inc. (Boulder, CO). After 12 h, cells were fixed for 30 min at 4°C in 4% formaldehyde dissolved in PEM buffer [400 mM Potassium PIPES, 0.5 M EGTA (pH 7.0), 1 M $MgCl_2$], washed $\times$3 with PEM buffer, treated in 0.1 M NH_4Cl (to quench autofluorescence), incubated for 30 min at room temperature in PEM + 0.5% Triton X-100, and washed again in PEM $\times$3. 4′,6-Diamidino-2-phenylindole dihydrochloride (Molecular Probes) staining was then performed using a 1 mg/ml stock diluted 2000-fold for 1 min. After mounting the coverslip, cells were observed under a fluorescence microscope. Cells showing green fluorescence were then ob-

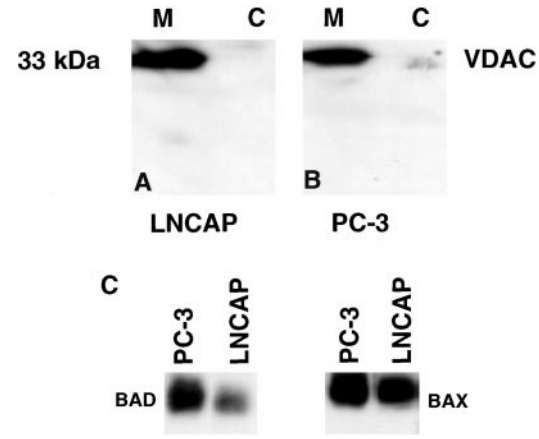

Fig. 3. Subcellular localization of VDAC in PC-3 and LNCaP cells. *A* and *B*, 10 μg of cell lysates obtained from each subcellular fraction were sized and processed as described in "Materials and Methods" for VDAC detection. A protein of the expected size is localized in the mitochondrial fraction of both cell lines. *C*, cell lysates were immunoprecipitated as described in "Materials and Methods" using the anti-VDAC antibody. Immunoprecipitates were then subjected to Western analysis using antibodies for Bax and Bad.

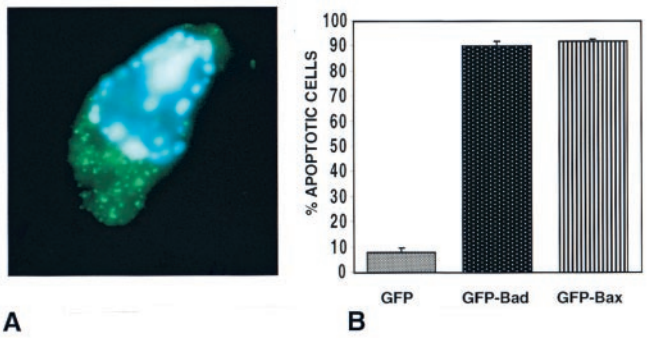

Fig. 4. Transient transfection of GFP-Bad and GFP-Bax but not of GFP is associated with dramatic induction of apoptosis in PC-3 cells. Cells were transfected overnight using the calcium precipitation transfection method and 1 μg of GFP-Bad, GFP-Bax, and GFP. Cells showing green fluorescence were analyzed for the presence of the typical apoptotic morphology shown in *A* (a PC-3 cell transfected with GFP-Bax). *B*, 300 cells from six different fields were scored in three experiments. Percentage of apoptotic cells in these three experiments are expressed as mean ± SD.

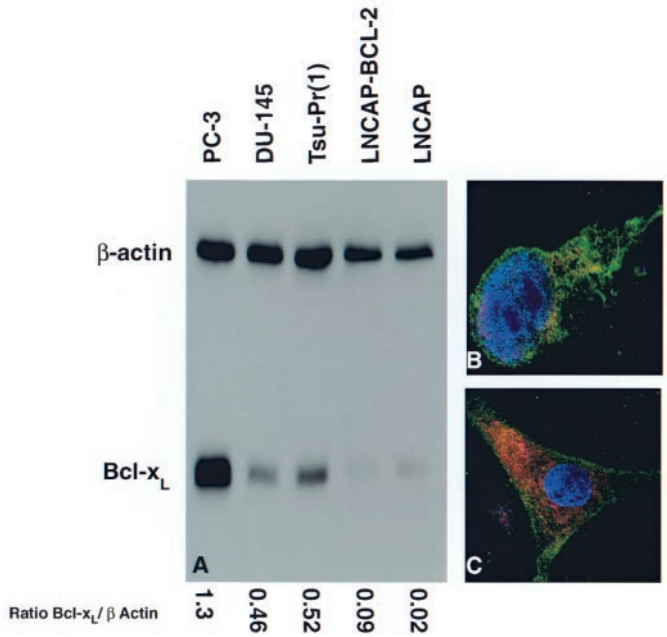

Fig. 5. Overexpression of Bcl-X_L in PC-3 cells. *A*, cell extracts (10 μg) were sized and subjected to Western analysis with antibodies for β-actin and Bcl-X_L. Arbitrary densitometric units of the Bcl-X_L band were corrected for those of the β-actin band as explained in "Materials and Methods." The ratio is reported at the bottom of each lane. *B* and *C*, confocal analysis for Bax (green fluorescence) and Bcl-X_L (red fluorescence) expression in LNCaP (*B*) and PC-3 cells (*C*).

served at higher magnification, and visualization of polyfragmented nuclei was interpreted as presence of apoptosis. A total of 300 cells in six different fields were scored for the presence (or absence) of apoptosis in each transfection.

Antisense Experiments. To create a PC-3 cell line expressing a reduced amount of Bcl-X_L, two phosphorothioate oligonucleotides were synthesized as described in the paper of Ackermann *et al.* (38). Oligonucleotide Bcl-X_L-AS ($^{5'}$CTACGCTTTCCACGCACAGT$^{3'}$) consists of nucleotides 581 to 601 of the Bcl-X_L coding sequence (39) in the antisense orientation. The mismatch oligonucleotide Bcl-X_L-MS ($^{5'}$CGACACGTACCTCTCGCATT$^{3'}$) has 60% homology to Bcl-X_L-AS. Oligos were transfected overnight at increasing concentrations (1 to 500 nM) using LipofectAMINE plus (25 μl in 100 μl of OptiMEM). Cells were harvested 48 h after transfection, and the expression of Bcl-X_L was evaluated using the quantitative Western analysis described above. Once conditions causing reproducible decrease of Bcl-X_L were identified, cells were grown and transfected with oligos Bcl-X_L-AS or Bcl-X_L-MS in duplicate dishes. At 48 h after transfection, one dish was harvested, and Western analysis of Bcl-X_L was performed to control its down-regulation. The other dishes were treated with 4 μM STS for 24 h. The presence of apoptosis was verified using the Cellular DNA Fragmentation ELISA kit from Boehringer.

RESULTS

To evaluate the integrity of the postmitochondrial machinery, cytosols from PC-3 and LNCaP cells were incubated in the presence and absence of bovine heart cytochrome *c* and dATP using the cell-free assay of Liu *et al.* (20). When all of these components were present, several hallmarks of apoptosis were evident, including cleavage of caspase-3, caspase-7, and DFF (Fig. 1). These results indicated that the postmitochondrial phase of the mitochondrial pathway of apoptosis was intact and functional in the two cell lines and that the locus of resistance to STS-induced apoptosis in PC-3 cells was at the premitochondrial or mitochondrial level.

Intracellular Trafficking of Apoptotic Signaling Molecules. Because the subcellular localization and proteolytic processing of members of the Bcl-2 family, the caspase family, and cytochrome *c* are critical to regulating proper engagement of the apoptotic machinery, we evaluated the potential role of these molecules in mediating apoptotic responses. In LNCaP cells (Fig. 2), the proapoptotic Bid was in the cytoplasm in the basal state and nearly completely proteolyzed in response to STS, most likely after caspase-dependent cleavage at one of its aspartate residues (40, 41). In PC-3 cells, Bid was unaffected by STS. In both lines, Bad and Bax translocated from the cytoplasm to the mitochondria with STS. Although Bcl-X_L was insensitive to STS in both lines, it was almost exclusively mitochondrial in LNCaP cells but equally distributed in PC-3 mitochondria and cytoplasm. As we reported previously (5, 27), cytochrome *c* translocated from the mitochondria to the cytoplasm in LNCaP cells, leading to cleavage of caspases-9, -3, and -7, independent of their subcellular localization. Conversely, these postmitochondrial mediators were STS-insensitive in PC-3 cells.

Molecular Analysis of STS Resistance in PC-3 Cells. On the basis of the above data, we deduced that the locus of resistance to STS was at the mitochondrial phase. Sequence analysis of Bax and Bad demonstrated that both these molecules were wild type in PC-3 cells. The only abnormality consisted in a polymorphism-changing codon 241 of Bad from CCT to CCA (Pro → Pro). Thus, to identify the

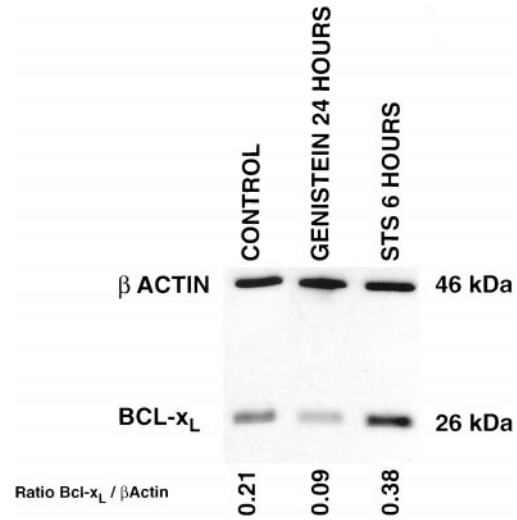

Fig. 6. Genistein decreases Bcl-X_L expression. Cells were treated with genistein (300 μM) for 24 h or STS (4 μM) for 6 h, harvested, and analyzed by Western analysis for Bcl-X_L and β-actin expression using 10 μg of cell lysates. Arbitrary densitometric units of the Bcl-X_L band were corrected for those of the β-actin band, and the ratio is reported at the bottom of each lane. A representative experiment of three is shown.

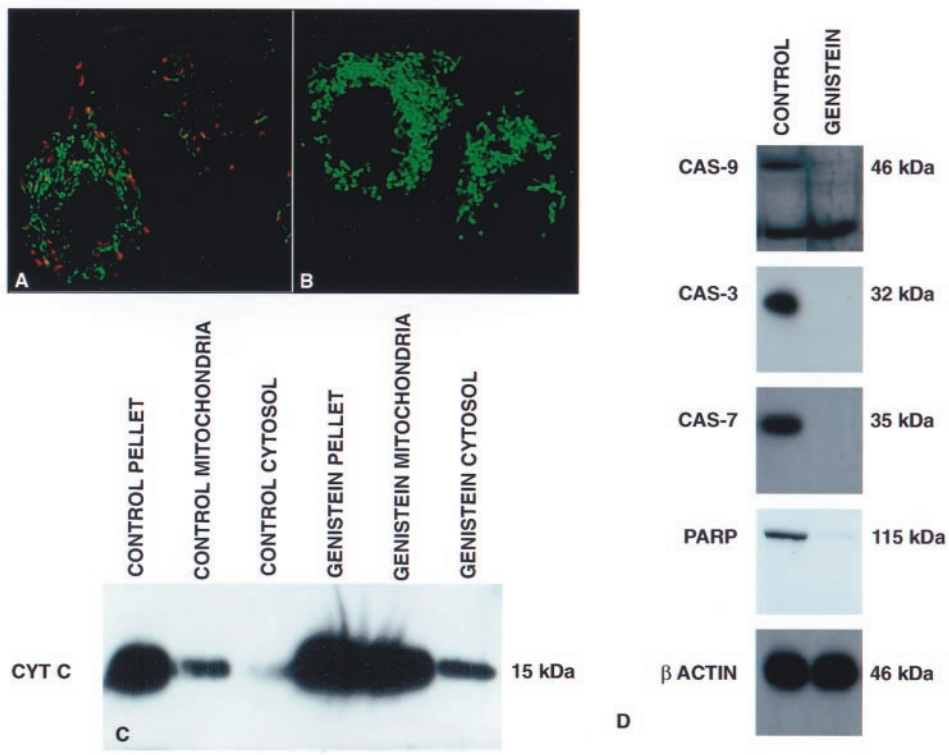

Fig. 7. Genistein induces apoptosis in PC-3 cells by activating the mitochondrial pathway. Cells were treated with genistein (300 μM) for 24 h, harvested, and analyzed for mitochondrial transmembrane potential (*A*, baseline; *B*, 24 h after genistein), cytochrome *c* subcellular localization (*C*), and procaspase-9, -3, -7, and PARP processing before and 24 h after genistein (*D*). β-actin expression was determined to demonstrate that a similar amount of cell extracts (10 μg) were loaded in each experiment (*D*). *C*, the various subcellular fractions represent the low-speed pellet (containing unbroken nuclei and cells) and the mitochondrial and cytosolic fractions, which were obtained as described in "Materials and Methods."

precise molecular defect, we investigated the expression and functional integrity of the VDAC (42) and the ANT (43), key mediators of the mitochondrial permeability transition pore. In both cell lines, VDAC (Fig. 3, *A* and *B*) and ANT (data not shown) were of the appropriate molecular weight and uniquely localized to the mitochondria (Fig. 3, *A* and *B*). VDAC coimmunoprecipitated with Bax and Bad, indicating normal functional interactions (Fig. 3*C*). Further, overexpression of VDAC had no effect on PC-3 cell sensitivity to STS (data not shown).

Overexpression of Bad and Bax Induces Apoptosis in PC-3 Cells. Next, we overexpressed GFP-Bax and -Bad to determine whether the mitochondrial phase of PC-3 cells was actually intact and could be forcibly engaged or if some other defect existed. Cells showing GFP fluorescence were scored as normal or apoptotic based on their morphology (Fig. 3*A*) and were found to be apoptotic in 90 and 92% of the cases after transfection with either GFP-Bax or GFP-Bad (Fig. 4). In contrast, only 6% of the cells transfected with the control plasmid expressing only the GFP protein were apoptotic (Fig. 4). These results confirmed that in PC-3 cells the mitochondrial apoptotic program could be activated if properly stimulated by proapoptotic Bcl-2 family members. In addition, these data suggested that the components of the mitochondrial permeability transition pore were able to mediate mitochondrial incapacitation after the interaction with proapoptotic Bcl-2 family members such as Bax and Bad. Subsequently, a systematic analysis of Bcl-X_L, Bcl-2, Bcl-w, mcl-1, and A1, molecules known to prevent apoptosis by protecting the mitochondria, was initiated in several prostate cancer cell lines. Most notably, Bcl-X_L expression was 45-fold greater in PC-3 compared with LNCaP cells (Fig. 5*A*). Overexpression of Bcl-X_L was also confirmed in experiments of immunofluorescence as shown in Fig. 5, *B* and *C*, where Bcl-X_L (red fluorescence) is significantly more expressed in PC-3 (Fig. 5*C*) compared with LNCaP cells (Fig. 5*B*).

Down-Regulation of Bcl-X_L Restores Sensitivity to STS-induced Apoptosis in PC-3 Cells. Genistein, a nonspecific inhibitor of protein tyrosine kinases, was reported previously (44) to down-regulate Bcl-X_L expression. In PC-3 cells, whereas STS actually increased Bcl-X_L about 2-fold, genistein caused a 2.3-fold decrease (Fig. 6). Further, genistein alone induced apoptosis by causing loss of mitochondrial transmembrane potential, release of cytochrome *c* to the cytosol, activation of the caspase pathway, and cleavage of the caspase target PARP (Fig. 7).

Butyric acid, which also was reported to down-regulate Bcl-X_L expression (45), caused a 7.3-fold reduction in Bcl-X_L protein (Fig. 8*A*). However, this was associated with only minimal cytochrome *c* translocation to the cytosol (compare Fig. 8*B* [baseline] *versus* Fig. 8*C* [after 48 h of butyric acid]) and minor cleavage of caspase-9 and caspase-7 or DFF (Fig. 8*E*). Nonetheless, significant cleavage of caspase-3 (Fig. 8*E*) was accompanied by a 15-fold induction of DEVDase (data not shown) and 37% TUNEL positivity (Fig. 8, *H* and *I*). Most importantly, butyric acid treatment conferred STS sensitivity to PC-3 cells as evidenced by additional down-regulation of Bcl-X_L (Fig. 8*A*), cytochrome *c* translocation from mitochondria to cytoplasm (compare Fig. 8*B* [baseline] with Fig. 8*D* [after 48 h of butyric acid + STS from 24 to 48 h]), complete cleavage of caspases-9, -3, and -7 as well as DFF (Fig. 8*E*), 18.5-fold increased DEVDase activity (data not shown), and massive (96%) induction of TUNEL positivity (Fig. 8, *F* and *G*).

Finally, antisense oligonucleotides were used to decrease Bcl-X_L expression in PC-3 cells. Antisense treatment for 24 h decreased Bcl-X_L protein ~4-fold compared with a mismatched control oligo and resulted in a 6-fold increase in apoptosis (Fig. 9).

DISCUSSION

The data presented here illustrate clear molecular differences in the susceptibility of different prostate cancer cell lines to undergo apoptosis. Whereas LNCaP cells engaged many of the typical components of the apoptotic machinery, PC-3 cells were extremely resistant to apoptosis. Using a cell-free system to reconstitute the postmitochondrial phase, it was evident that the two lines were equally capable of

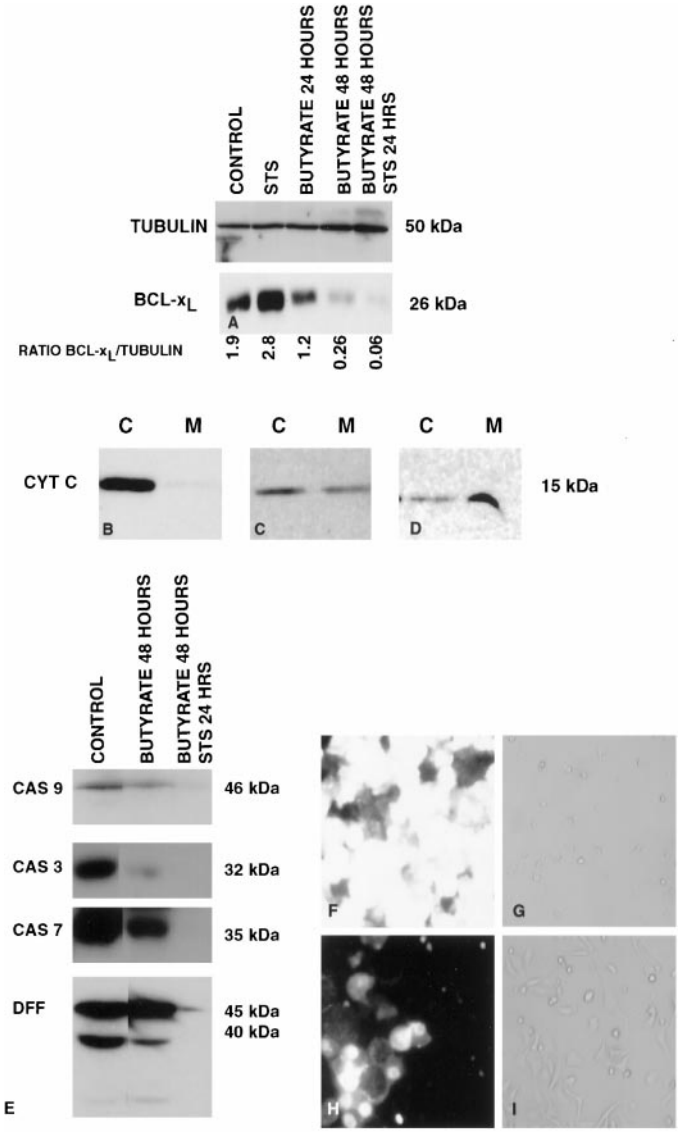

Fig. 8. Sodium butyrate sensitizes PC-3 cells to the apoptotic effect of STS. Cells were treated with vehicle alone, butyrate (2 mM for 24 h), butyrate (2 mM for 48 h), or butyrate (2 mM for 48 h) + STS 4 μM (for the last 24 h of the experiment). *A*, cell lysates (10 μg) were subjected to Western analysis for Bcl-X_L or tubulin. Arbitrary densitometric units of the Bcl-X_L band were corrected for those of the tubulin band, and the ratio is reported at the bottom of each lane. Cytochrome *c* subcellular localization in (*B*) control, (*C*) butyrate (2 mM for 48 h), or (*D*) butyrate (2 mM for 48 h) + STS (4 μM from the 24th to 48th h) treated cells. Equal amounts (4 μg) of mitochondrial (*M*) or cytosolic (*C*) subfractions were sized and analyzed for cytochrome *c* expression. *E*, cells were analyzed for procaspase processing and DFF cleavage. *F-I*, TUNEL (adherent and floating cells; *F*, *H*) and phase contrast (adherent cells; *G*, *I*) analysis of cells treated with STS + butyrate (*F*, *G*) or butyrate alone (*H*, *I*). Note that most cells are TUNEL positive after STS + sodium butyrate (*F*), corresponding to cell loss from the plate (*G*).

engaging this phase of apoptosis. In addition, both lines exhibited cytoplasm-to-mitochondrial translocation of Bad and Bax, thus indicating the premitochondrial phase was intact and functional.

On the basis of these observations, we focused on the mitochondrial phase as the locus of apoptosis resistance in PC-3 cells. Sequence analysis indicated that Bax and Bad did not contain any defective mutations and overexpression of either of these proapoptotic molecules induced apoptosis. Further, VDAC and ANT, key components of the mitochondrial permeability transition pore, were of the correct size and in the appropriate mitochondrial subfraction. VDAC overexpression in stably transfected PC-3 cells also failed to increase sensitivity to STS-induced apoptosis. These observations confirmed that the components of the permeability transition pore were functionally normal and suggested that an antiapoptotic factor with the ability to protect the mitochondria was overexpressed in PC-3 cells. Four lines of evidence support the hypothesis that Bcl-X_L overexpression is responsible for the resistance of the PC-3 cells to STS-induced apoptosis: *a*.) Bcl-X_L was overexpressed in PC-3 cells relative to several apoptosis-sensitive prostate cell lines; *b*.) genistein, a tyrosine kinase inhibitor, down-regulated Bcl-X_L and induced spontaneous apoptosis; *c*.) sodium butyrate down-regulated Bcl-X_L expression and, most importantly, conferred STS sensitivity; and *d*.) antisense down-regulation of Bcl-X_L restored STS-induced apoptosis. These results indicated that elevated Bcl-X_L was responsible for protecting PC-3 cells from apoptosis.

Overexpression of Bcl-X_L has already been described in PC-3 cells (46) and in cell lines manifesting multiple drug resistance (47, 48). In the latter, overexpression of Bcl-X_L is associated with apoptosis-resistance because of abrogation of cytochrome *c* release to the cytosol. Bcl-X_L overexpression does not functionally substitute for a mutagenic initiator or mitogenic promoter in tumorigenesis. However, there is an increased potential for benign tumors overexpressing Bcl-X_L to undergo malignant degeneration possibly because of their prolonged survival (49). The mechanism leading to apoptosis-resistance after Bcl-X_L overexpression has to do with its ability to prevent cytochrome *c* translocation to the cytosol and to protect the mitochondria from undergoing functional incapacitation (50). This is in part achieved through inactivation of proapoptotic Bcl-2 family members via heterodimerization (51, 52) and in part by facilitating mitochondrial ATP/ADP exchange (53).

The signaling pathway regulating Bcl-X_L expression is only partially understood. Using murine myeloid cell lines, Bcl-X_L expression was found to be positively regulated by IFN-γ (54) and through a Jak

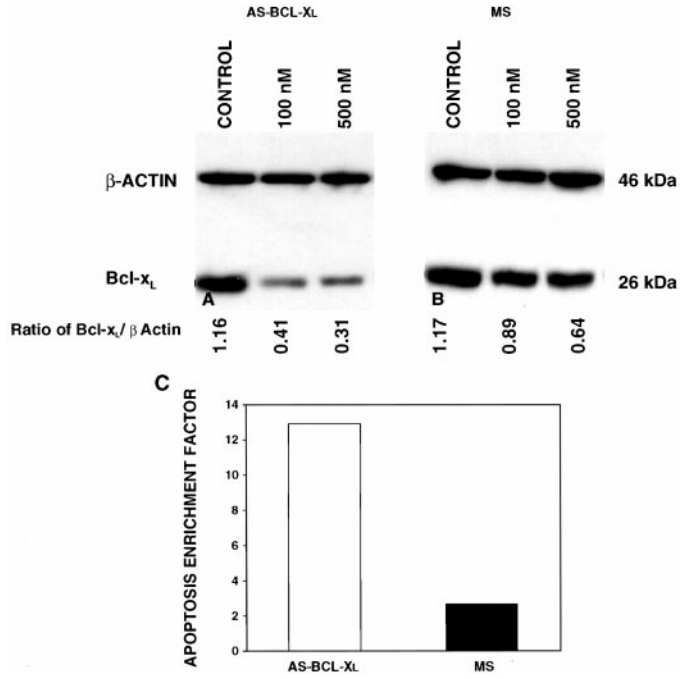

Fig. 9. Treatment with Bcl-X_L antisense (*A*), but not with mismatch (*MS*) oligonucleotides (*B*), is associated with decreased expression of Bcl-X_L protein. Cells were transfected overnight with increasing concentrations of oligos and analyzed by Western analysis with antibodies for β-actin and Bcl-X_L. Arbitrary densitometric units of the Bcl-X_L band were corrected for those of the β-actin band, and the ratio is reported at the bottom of each lane. *C*, only cells treated with the Bcl-X_L oligo resume sensitivity to STS-induced apoptosis by undergoing DNA laddering. Cells were transfected with 500 nM of AS-Bcl-X_L or mismatch and then treated for 24 h with STS (4 μM). DNA laddering was measured using the Cellular DNA Fragmentation ELISA kit from Boehringer, as described in "Materials and Methods." A representative experiment of three is shown.

kinase-dependent interleukin 3 pathway (55). Other investigators have identified a connection between induction of Bcl-X_L expression and the activity of tyrosine kinase receptors such as HER2 (13, 56) and a tumor-specific mutant of epidermal growth factor receptor (57). The fact that the tyrosine kinase inhibitor, genistein, decreased Bcl-X_L expression in PC-3 cells supports the conclusion that overexpression of Bcl-X_L could be mediated through this signaling pathway. Another mechanism of Bcl-X_L regulation is by inactivation through posttranslational modifications. For instance, in response to genotoxic agents, the stress-activated protein kinase translocates to the mitochondria and phosphorylates Bcl-X_L, presumably leading to its inactivation (58).

Upon demonstrating Bcl-X_L overexpression in PC-3 cells, we wondered if this is a common occurrence in other prostate cancer cell lines or in clinical specimens from patients with prostate cancer. Other prostate cancer cell lines such as DU-145 and TSU-Pr (1) express higher concentrations of Bcl-X_L than LNCaP, although not to the same extent as PC-3 (Fig. 5). Tissue from patients with prostate cancer has not been investigated for its presence of Bcl-X_L overexpression, despite the fact that since 1992 an extensive literature has identified a potential connection between the close relative Bcl-2 and hormone-independent prostate cancer (59, 60). In view of the observations reported in this study and of the well-established role of Bcl-X_L as one of the most powerful antiapoptotic factors, studies addressing the correlation between Bcl-X_L expression and the various phases of prostate cancer are now warranted. In addition, it will be important to understand the details of the signaling pathway mediating Bcl-X_L overexpression in PC3. This will help with the identification of new potential therapeutic targets to decrease the threshold of apoptosis-sensitivity in prostate cancer.

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