

# Bcl-xL Is Overexpressed in Hormone-Resistant Prostate Cancer and Promotes Survival of LNCaP Cells via Interaction with Proapoptotic Bak

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**Androgen-sensitive prostate cancer cells turn androgen resistant through complex mechanisms that involve dysregulation of apoptosis. We investigated the role of antiapoptotic Bcl-xL in the progression of prostate cancer as well as the interactions of Bcl-xL with proapoptotic Bax and Bak in androgen-dependent and -independent prostate cancer cells. Immunohistochemical analysis was used to study the expression of Bcl-xL in a series of 139 prostate carcinomas and its association with Gleason grade and time to hormone resistance. Expression of Bcl-xL was more abundant in prostate carcinomas of higher Gleason grades and significantly associated with the onset of hormone-refractory disease. *In vivo* interactions of Bcl-xL with Bax or Bak in untreated and camptothecin-treated LNCaP and PC3 cells were investigated by means of coimmunoprecipitation. In the absence of any stim-**

**uli, Bcl-xL interacts with Bax and Bak in androgen-independent PC3 cells but only with Bak in androgen-dependent LNCaP cells. Interactions of Bcl-xL with Bax and Bak were also evidenced in lysates from high-grade prostate cancer tissues. In LNCaP cells treated with camptothecin, an inhibitor of topoisomerase I, the interaction between Bcl-xL and Bak was absent after 36 h, Bcl-xL decreased gradually and Bak increased coincidentally with the progress of apoptosis. These results support a model in which Bcl-xL would exert an inhibitory effect over Bak via heterodimerization. We propose that these interactions may provide mechanisms for suppressing the activity of proapoptotic Bax and Bak in prostate cancer cells and that Bcl-xL expression contributes to androgen resistance and progression of prostate cancer. (*Endocrinology* 147: 4960–4967, 2006)**

**T**HERAPY AGAINST ADVANCED prostate cancer usually involves androgen ablation because this efficiently induces tumor regression (1). However, prostate cancer cells turn androgen resistant in most cases shortly after the onset of ablative therapy. At this stage, other conventional anticancer therapies are also poorly effective, and therefore, understanding the molecular basis of androgen resistance and prostate cancer progression is crucial to the development of more effective regimes for advanced disease. Androgen deprivation reduces tumor growth by decreasing cell proliferation and promoting apoptotic cell death. Progression of localized hormone-dependent prostate cancers to metastatic hormone-refractory disease is also associated with dysregulation of normal apoptotic mechanisms (2). Overexpression of Bcl-2 in prostate carcinoma cells is a mechanism proposed for advanced and hormone-refractory disease (3, 4). Nonetheless, the clinical significance of Bcl-2 in human prostate cancer remains controversial (5, 6).

The Bcl-2 family proteins have an essential role in the mitochondrial pathway of apoptosis. This family includes both proapoptotic and antiapoptotic members. In fact, the interactions between these subpopulations determine, at

least in part, the susceptibility of cells to a death signal. The Bcl-2 family proteins fall into three groups that share at least one of four conserved Bcl-2 homology domains (BH1–4). Bcl-2, Bcl-xL, Bcl-w, A1, and Mcl-1 bear three or four BH domains and promote cell survival. Bcl-xL is a potent antiapoptotic factor that mediates resistance to staurosporin-induced apoptosis in PC3 cells and apoptosis can be restored when Bcl-xL is down-regulated (7, 8). Bcl-xL may neutralize the release of cytochrome c (9), but the exact mechanism by which Bcl-xL exerts its antiapoptotic effect remains controversial (10). Multidomain proapoptotic proteins Bax, Bak, and Bok share three domains (BH1–3) with Bcl-2 and are essential mediators in the apoptotic process. In mouse embryonic fibroblasts the absence of Bax and Bak is necessary for the effectiveness of the apoptotic blockade (11). Bax overexpression resulted in apoptotic cell death in prostate cancer cells such as PC3 and DU145, which are known to offer resistance to a variety of chemical proapoptotic agents (8, 12). The third group is constituted by the diverse group of proapoptotic BH3-only proteins, including Bad, Bim, Bid, Puma, and Noxa, which have in common only the small BH3 interaction domain (13). These proteins control the activation of multidomain proapoptotic proteins, but the exact mechanism remains unclear. After an apoptotic signal, BH3-only proteins could promote multidomain proapoptotic proteins activation via their ability to inactivate the Bcl-2-like proteins. Alternatively, Bax may be activated via direct engagement by certain BH3-only proteins, the best documented being the active truncated form of Bid, tBid (14–16).

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Abbreviations: CHAPS, 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate; PARP, poly(ADP-ribose) polymerase; PSA, prostate-specific antigen.

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These models can explain how multidomain proapoptotic proteins are activated on a death stimulus; however, the mechanism by which Bax and Bak are kept in an inactive state in untreated cells remains a matter of discussion. In this regard, it has been published the interaction between Bak with Bcl-xL in untreated HeLa (17) and PC3 cells (18). The case of Bax is more controversial. Several authors state that Bax is predominantly a cytosolic monomer in untreated cells (19); during apoptosis it undergoes conformational changes, translocates to the outer mitochondrial membrane, and forms oligomers that cause mitochondrial dysfunction and apoptotic cell death (20–23). On the other hand, the interaction between Bax and Bcl-xL in untreated T24 (24) and KB-3 cells (25) has been published recently. Also, it has been proposed that several unrelated proteins modulate Bax activity via direct interaction in untreated cells, such as Bif-1, 14–3–3 $\theta$ , Hsp60, and Ku70 (26–29). Thus, whether the antiapoptotic proteins restrain Bax activation directly or indirectly remains uncertain (30) because it seems to be highly dependent on the cellular type.

Our aim was first to investigate whether proteins with suppressor activity on proapoptotic Bax and Bak, like Bcl-xL, were overexpressed in prostate cancer tissues and study their association with hormone-resistant, high-grade phenotype. Moreover, we intended to elucidate whether Bcl-xL actually interact with proapoptotic Bax and Bak in prostate cancer cell lines and tissues and thereby support that their blockade by Bcl-xL has a role in the prevention of apoptosis and the progression of hormone-resistant prostate cancer.

## Materials and Methods

### Patients and tumor tissues

One hundred thirty-nine patients with prostate cancer were selected, 45 of them eligible for androgen ablation therapy (*i.e.* locally advanced carcinoma stages T3c–T4, C2–C3; or carcinoma with evidence of metastatic disease N+/M+). This group of patients was followed up during a period of 48 months since the beginning of the treatment. Informed consent was required from patients according to the policies of the ethical committee of our institution. Exclusion criteria were evidence of concomitant inflammatory disease of the prostate, urolithiasis, or the presence of permanent vesical catheterization. Pathological diagnosis was reported on echography-directed transrectal biopsies. Patients were followed up every 3 months in a specialized prostate cancer unit at our hospital. Tumors were defined as androgen dependent or androgen independent after 3 months of complete androgen blockade therapy that consisted of 50 mg/d bicalutamide during 2 wk and 22.5 mg leuprolide acetate given im every 3 months until the appearance of hormone resistance. Androgen-dependent tumors were defined according to the criteria of symptom relief and more than 50% decrease of serum prostate-specific antigen (PSA) levels at diagnosis. Conversion to androgen-independent status was defined whenever there was an increase in two consecutive serum PSA determinations, increase of tumor size, or the appearance of *de novo* metastases. Tissue biopsies were fixed in 10% buffered formalin and embedded in paraffin blocks following standard procedures. At least one unfixed tumor tissue cylinder was snap frozen in liquid nitrogen and stored at –80 C until use. Two independent pathologists evaluated Gleason grading according to conventional criteria. Gleason grade low is 2–4, moderate 5–7, and high 8–10 (31). For statistical analysis, Gleason grades were categorized into grades 2–6 and 7–10.

### Cell culture

Human prostate cancer cell lines PC3 and LNCaP-FNC were obtained from the Interlab Cell Line Collection (Genoa, Italy) and routinely grown

in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 1 mM glutamine in a 37 C, humidified incubator under 5% CO<sub>2</sub>. Subconfluent cell cultures were harvested by trypsinization.

### Antibodies

Rabbit polyclonal anti-Bax and anti-Bak, and mouse monoclonal anti-Bcl-xL antibodies were available from BD PharMingen (San Diego, CA), a second mouse monoclonal anti-Bcl-xL antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal anti- $\beta$ -actin from Sigma (St. Louis, MO). Dilutions used in Western blots were anti-Bax (1:1000); anti-Bak (1:2000); anti-Bcl-xL (1:100; Santa Cruz Biotechnology); and anti- $\beta$ -actin (1:5000). For immunohistochemical procedures, dilutions were anti-Bax (1:2000) and anti-Bcl-xL (BD PharMingen) (1:1000).

### Western blotting

Forty micrograms (from cell lines) or 100  $\mu$ g (from tissues with tumor cell content over 75%) of total protein, as determined by using BCA protein assay kit (Pierce, Rockford, IL), were separated by SDS-PAGE on 4–20% gradient polyacrylamide gels (Invitrogen, Carlsbad, CA). Gels were electroblotted onto nitrocellulose membranes (Amersham, Little Chalfont, UK). For immunodetection, blots were blocked in 1% blocking reagent (Roche, Mannheim, Germany) in 0.05% Tween 20-PBS for 1 h and incubated with primary antibody overnight at 4 C diluted in blocking buffer. Blots were then washed in 0.05% Tween 20-PBS and incubated with either goat antimouse (1:10,000; Amersham) or goat anti-rabbit (1:20,000; Amersham) peroxidase-labeled antibodies in blocking buffer for 1 h. Enhanced chemoluminescent system was applied according to the manufacturer's protocol (Amersham). Scanning densitometry was performed with Scan Analysis software (Biosoft, Cambridge, UK). Arbitrary densitometric units of the protein of interest were corrected for those of  $\beta$ -actin.

### Immunohistochemistry

Five-micrometer-thick tissue sections from paraffin blocks were dewaxed and rehydrated. Sections were immersed in 3% H<sub>2</sub>O<sub>2</sub> aqueous solution for 30 min to exhaust endogenous peroxidase activity and then covered with 10% normal swine serum in Tris-buffered saline to block nonspecific binding sites. Antigen retrieval was performed with a pressure cooker, using citrate buffer (pH 6.0 for Bax) or 10 mM Tris and 1 mM EDTA (pH 9.0 for Bcl-xL). Bak immunohistochemistry was performed without antigen retrieval. Sections were incubated with primary antibodies overnight at 4 C. Peroxidase-labeled secondary antibodies and 3,3'-diaminobenzidine were applied to develop immunoreactivity, according to manufacturer's protocol (EnVision; Dako, Glostrup, Denmark). Slides were then counterstained with hematoxylin and mounted in DPX (BDH Laboratories, Poole, UK). Sections in which primary antibody was omitted were used as negative controls. Immunostaining was evaluated independently by two observers on at least 10 microscopic fields at magnification  $\times$ 200 and scored as follows: 2+, greater than 50%; +, greater than 5%; and –, 1–5% of the carcinoma cells immunostained.

### Immunoprecipitations

LNCaP and PC3 cells or tumor tissues were lysed in 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) buffer [20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.25 M sucrose, and 2% CHAPS], containing protease inhibitors (1:100 dilution of protease inhibitors cocktail, catalog no. P8340; Sigma). One to two milligrams of total lysate were incubated with either 80  $\mu$ l protein A-Sepharose beads (Amersham) for polyclonal antibodies or 80  $\mu$ l of protein G-Sepharose beads (Amersham) for monoclonal Bcl-xL antibody at 4 C for 3 h. After centrifugation, beads were discarded and supernatants were incubated with normal rabbit serum for polyclonal antibodies or mouse IgG<sub>1</sub> for anti-Bcl-xL overnight at 4 C, followed by protein A or G-Sepharose beads for 2 h. After centrifugation, beads were discarded and supernatants were incubated at 4 C for 1 h in a rotator with anti-Bax polyclonal antibody, anti-Bak polyclonal antibody, anti-

Bcl-xL monoclonal antibody, and normal rabbit serum or mouse IgG<sub>1</sub> as negative controls. Immunocomplexes were precipitated with 40  $\mu$ l of protein A or G-Sepharose beads for 1 h at 4 C. After eight washes in CHAPS buffer, beads were boiled in 15  $\mu$ l Laemmli buffer and subjected to SDS-PAGE. Normal rabbit serum and mouse IgG<sub>1</sub> were from Dako.

#### Apoptosis induction and assays

Stock solution of camptothecin (Sigma) was prepared at 100 mM and stored frozen. Subconfluent monolayers of cells were treated with 3  $\mu$ M camptothecin in culture medium for 18 and 36 h. One million cells were washed in cold PBS and suspended in 100  $\mu$ l Annexin V binding buffer (R&D Systems, Minneapolis, MN) containing 5  $\mu$ g/ml propidium iodide and 0.5  $\mu$ g/ml annexin V-fluorescein isothiocyanate, incubated for 15 min at room temperature in the dark, and diluted in 400  $\mu$ l Annexin V binding buffer. Fluorescence was measured on a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA) within 1 h. The collected events were gated on the forward and side scatter plots to exclude cellular debris. Three discrete cell populations (viable, early apoptotic, and late apoptotic) were identified measuring fluorescence on FL1 and FL2 channels. Annexin V binding assays were repeated three times in independent experiments. Caspase-mediated cleavage of poly-(ADP-ribose) polymerase (PARP) was assessed by Western blot using a monoclonal antihuman PARP (BD PharMingen) at 1:250 dilution.

#### Statistics

Association of Bcl-xL with clinicopathological variables was analyzed by Fisher's exact test. Hormone-refractory, disease-free survival curves were calculated by the method of Kaplan and Meier. Comparison of survival curves was done by the log-rank test of Mantel and Haenszel. Calculations were performed using Prism 4.0 (GraphPad, San Diego, CA).

### Results

#### *Bcl-xL is overexpressed in high-grade prostate carcinoma and associates with hormone-refractory phenotype*

To characterize the expression of Bcl-xL, Bak, and Bax proteins in human prostate cancer, we performed an immunohistochemical analysis of 139 formalin-fixed and paraffin-embedded tissue biopsies. Representative micrographs of the findings obtained after immunoperoxidase staining for Bcl-xL are shown in Fig. 1, A–C. Well-differentiated, low Gleason-grade prostatic carcinomas showed very low levels of Bcl-xL, in contrast to tumors of moderate to high Gleason grade that exhibited increasing amounts of Bcl-xL. The appearance of Bcl-xL immunoreactivity was coarsely granular, cytoplasmic staining. Some benign hyperplastic glands contained few immunoreactive cells, but the staining was far less intense and finely granular. Stromal cells did not show immunoreactivity for Bcl-xL. Immunostaining for Bax and Bak was detected in all prostatic carcinomas with no major differences according to Gleason grade (data not shown).

Expression of Bcl-xL, Bax, and Bak proteins was also investigated by Western blot analysis in human prostatic carcinoma tissues (Fig. 1D, top). In tissues from prostatic carcinomas, the levels of Bcl-xL protein expression increased with the Gleason grade because the signal intensity of the detected 30-kDa band was low in grades 3–6 and high in grades 7–9. In the same tissues, the expression levels of Bak and Bax proteins were similar in all samples tested and were not dependent on Gleason grade. Bax protein was detected as a double band in all prostatic carcinoma tissues. Average densitometric values of normalized Bcl-xL protein expression levels showed 4.5-fold increase in the group of tumors

of Gleason grades 7–9, compared with that of Gleason grades 3–6. The group of carcinomas of higher Gleason grades had slightly lower levels of Bak and Bax than carcinomas of lower grades (Fig. 1D, bottom). In addition, we performed a statistical analysis of immunohistochemical Bcl-xL expression that showed a significant association with Gleason grade ( $P < 0.0001$ ) (Table 1). Most (85.1%) low Gleason-grade tumors (grades 2–4) showed low levels of Bcl-xL expression. Intermediate Gleason grade tumors (grades 5–7) had low or moderate levels in 65.6% of tumors and high in 34.4% of tumors. Most tumors of Gleason grades 8–9 (93.8%) expressed high levels of Bcl-xL. Immunohistochemical expression of Bcl-xL was also associated with the presence of regional lymph node involvement, distant metastasis, and initial serum PSA levels (Table 1).

Finally, we performed the analysis of Bcl-xL expression in relation to the hormone-responsive status of patients with locally advanced disease. Forty-five patients were treated by complete androgen blockade and followed up every 3 months. Their diagnostic biopsies were analyzed for the expression of Bcl-xL by immunohistochemistry. We performed a Kaplan-Meier survival analysis classifying these tumors into two groups, high ( $n = 20$ ) and low/moderate ( $n = 25$ ), according to the expression of Bcl-xL, and evaluating the time in months until the appearance of hormone-independent phenotype as the time variable (Fig. 1E). Survival curves were significantly different ( $P < 0.0001$  from Mantel and Haenszel log-rank test;  $\chi^2 = 21.2$ ; degrees of freedom = 1) between both groups with a median survival time of 14 months and a hazard ratio of 9.71 (3.72–26.16; 95% confidence interval) for the high Bcl-xL-expressing tumors.

#### *Bcl-xL forms complexes with Bax and Bak in androgen-independent PC3 cells*

We used cell lysates from androgen-dependent LNCaP cells and androgen-independent PC3 cells to elucidate the mechanism by which proapoptotic potential of Bax and Bak are diminished in relation to hormone-resistant disease. Interactions of Bcl-xL, with proapoptotic Bax and Bak were analyzed in cell lysates obtained with CHAPS buffer. Figure 2A shows the basal levels of Bcl-xL, Bak, and Bax in PC3 and LNCaP cells. Bcl-xL protein was more abundant in PC3 than LNCaP cells. Conversely, Bax protein levels were higher in LNCaP cells. Immunoprecipitation experiments with these cell lysates showed that Bcl-xL was coimmunoprecipitated with both anti-Bax and anti-Bak polyclonal antibodies in PC3 but only with anti-Bak in LNCaP cells (Fig. 2B). Figure 2C shows a Western blot of immunoprecipitated Bax and Bak that demonstrates the specificity and efficiency of immunoprecipitation experiments. The reciprocal immunoprecipitation for Bcl-xL confirms the interaction between Bcl-xL and Bax in PC3 cells (Fig. 2D). These results suggest that Bax and Bak are subject to a blockade by Bcl-xL in the androgen-independent PC3 cells. To further investigate whether interactions between Bcl-xL and proapoptotic Bax and Bak are potential mechanisms involved in prostate cancer progression, we performed immunoprecipitation experiments with prostatic carcinoma tissue lysates. Figure 2E shows these experiments in two lysates from prostatic carcinoma tissues

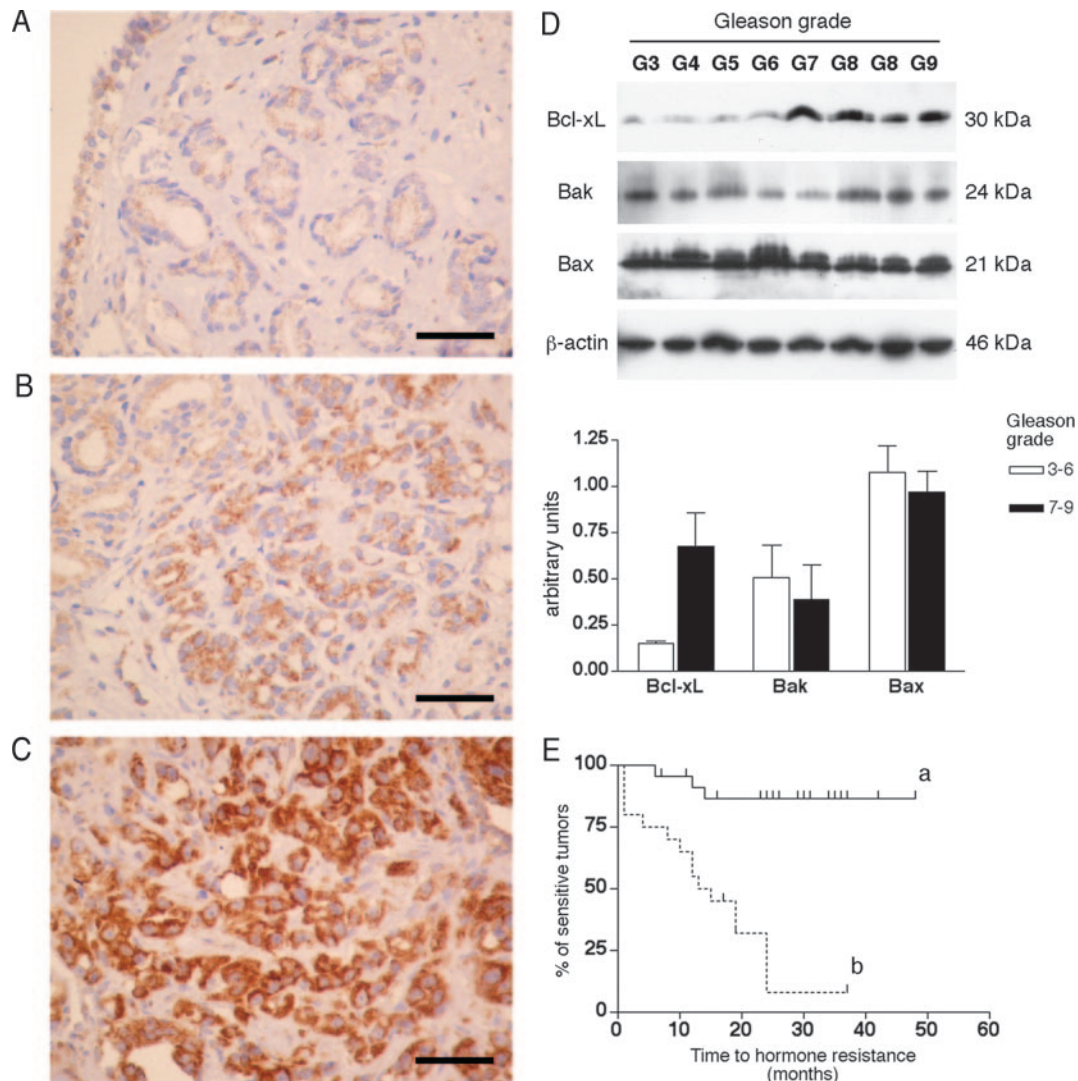


FIG. 1. Expression of Bcl-xL in prostatic carcinoma tissues. A–C, Immunoperoxidase analysis of Bcl-xL in formaldehyde-fixed, paraffin-embedded prostatic carcinoma tissues of low (A), moderate (B), and high (C) Gleason grades. Bar, 50  $\mu$ m. D, Western blot analysis of Bcl-xL, Bak, and Bax in eight prostatic carcinoma tissues of increasing Gleason grade. Densitometric analysis of Bcl-xL, Bak, and Bax expression levels normalized to  $\beta$ -actin expression levels is shown at the bottom. Average normalized values of each protein were calculated for Gleason grades 3–6 and Gleason grades 7–9 tumors. E, Kaplan-Meier analysis of hormone-refractory, disease-free survival in groups of patients with low/moderate (a, n = 25) or high (b, n = 20) levels of Bcl-xL expression. Follow-up period was 48 months. Tick marks represent censored patients. P value from log-rank test of Mantel and Haenszel.

of moderate and high Gleason grades (Gleason 5 and 9), respectively. Bcl-xL was coimmunoprecipitated with both anti-Bax and anti-Bak antisera in the lysate from prostatic carcinoma of high Gleason grade. In contrast, those interactions were not seen when using the lysate from moderately differentiated Gleason-grade prostatic carcinoma. These findings support the proposed mechanism of a blockade of Bax and Bak by Bcl-xL that might be involved in progression to hormone-refractory, high-grade prostate cancer.

#### Interaction between Bcl-xL and Bak is diminished after camptothecin treatment in LNCaP cells

To investigate the described interactions between Bcl-xL with Bax and Bak after an apoptotic stimulus, we treated PC3 and LNCaP cells with camptothecin, a topoisomerase I in-

hibitor. We measured PARP cleavage as a marker of induction of apoptosis (Fig. 3A). In LNCaP cells treated with 3  $\mu$ M camptothecin, the 85-kDa cleavage product appears at both 18 and 36 h, whereas PC3 cells show an intact PARP 36 h after the same treatment. Flow cytometry experiments with cells labeled with annexin V-fluorescein isothiocyanate and propidium iodide (Fig. 3B) confirm that PC3 cells are camptothecin insensitive. Conversely, LNCaP cells are camptothecin sensitive and undergo apoptosis upon the treatment. In this context, we examined the basal levels of Bcl-xL, Bak, and Bax in both cell lines, as well as the interactions between these proteins after treatment with 3  $\mu$ M camptothecin, to assess changes in the status of the interactions observed in untreated cells. In PC3 cells exposed to camptothecin during 36 h, Bak protein level is elevated, whereas the levels of

**TABLE 1.** Association of Bcl-xL immunohistochemical expression with selected clinicopathological variables of prostatic carcinomas

Variables	No. of patients	Bcl-xL expression			P value <sup>a</sup>
		Low	Moderate	High	
Tumor stage, n (%)					
T0–T2	104	42 (40.4)	35 (33.6)	27 (26.0)	0.001
T3–T4	26	5 (19.2)	5 (19.2)	16 (61.6)	
Lymph node metastasis, n (%)					
Yes	26	2 (7.7)	9 (34.6)	15 (57.7)	0.0008
No	71	37 (52.1)	20 (28.2)	14 (19.7)	
Distant metastasis, n (%)					
Yes	29	5 (17.2)	8 (27.6)	16 (55.2)	0.002
No	91	39 (42.8)	31 (34.1)	21 (23.1)	
Gleason grade, n (%)					
2–6	84	51 (60.7)	23 (27.3)	10 (12.0)	<0.0001
7–10	55	2 (3.6)	11 (20.0)	42 (76.4)	
Initial serum PSA level (ng/ml), n (%)					
0–10	41	22 (53.7)	13 (31.7)	6 (14.6)	0.002
>10	89	25 (28.1)	27 (30.3)	37 (41.6)	

<sup>a</sup> P value from Fisher's exact test. For the purpose of calculations Bcl-xL expression was categorized into low/moderate *vs.* high.

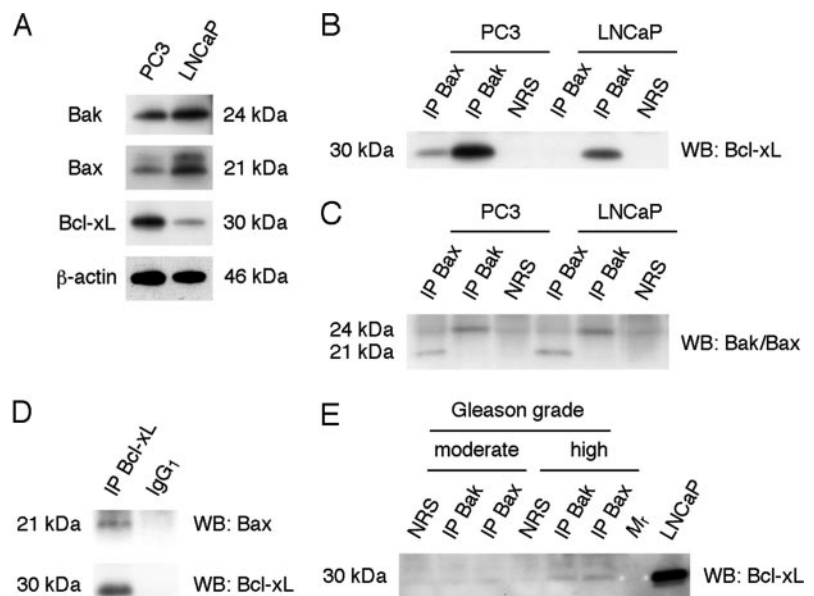
Bcl-xL and Bax proteins are slightly diminished, compared with control cells (Fig. 4A). Remarkably, these cells still maintain the interactions between Bcl-xL with Bax and Bak (Fig. 4B). In LNCaP cells exposed to the same stimulus, Bax and Bak protein levels are elevated; however, Bcl-xL protein expression is strongly down-regulated at 18 h and is almost undetectable at 36 h (Fig. 4A). According to the kinetics of Bcl-xL expression, the interaction between Bcl-xL and Bak is diminished at 18 h and have almost disappeared at 36 h in camptothecin-treated LNCaP cells (Fig. 4B); thus, Bak would be gradually free from Bcl-xL to exert its proapoptotic function.

### Discussion

Prostate cancer cells may use multiple molecular mechanisms to evade apoptosis, which along with increased proliferation contribute to extend survival. Prostate tumors respond to androgen ablation therapy by undergoing apoptotic cell death, but after a 12- to 18-month period, 50% of prostate cancer cells become androgen independent and

apoptosis resistant (32). The Bcl-2 family of proteins plays a critical role in the mitochondrial pathway of apoptosis. Multidomain proapoptotic molecules Bax or Bak are required to execute the mitochondrial pathway of apoptosis (11). Antiapoptotic proteins like Bcl-2 and Bcl-xL inhibit the release of certain proapoptotic factors from the mitochondria. We aimed to study Bcl-xL in human prostate cancer because it has a pivotal role in the survival of tumor cells (33, 34). Overexpression of Bcl-xL has been described in PC3 cells and cell lines manifesting multiple drug resistance (7, 8, 35). Tissues from patients with prostate cancer have not been specifically investigated for the presence of Bcl-xL protein. Krajewska *et al.* (5), in a study with an antibody against Bcl-x, showed a relation with the Gleason grade of primary prostate tumors. Elevated expression of Bcl-xL has been previously reported in some human malignancies including colorectal adenocarcinomas, Kaposi's sarcoma, and multiple myeloma (36–39). Endogenous high Bcl-xL expression is important for the inhibition of apoptosis triggered by various cellular stresses in hepatocellular carcinoma cell lines, such as stau-

**FIG. 2.** Immunoprecipitation of Bcl-xL with Bax and Bak in prostate cancer cells. **A**, Western blot analysis of Bcl-xL, Bak, and Bax basal expression levels in prostate cancer cell lines PC3 and LNCaP. **B**, Immunoprecipitation (IP) analysis of CHAPS-buffered lysates from PC3 and LNCaP cells using anti-Bax and anti-Bak polyclonal antibodies and normal rabbit serum (NRS) as negative control. Immunoprecipitates were analyzed by Western blot (WB) using an anti-Bcl-xL monoclonal antibody. **C**, Western blot of immunoprecipitated Bax and Bak. **D**, Reciprocal immunoprecipitation for Bcl-xL in PC3 cells. The lysate immunoprecipitated with anti-Bcl-xL and mouse IgG<sub>1</sub> as negative control was analyzed by Western blot with anti-Bax antibody. **E**, Immunoprecipitation of Bax and Bak in prostatic carcinoma tissues. CHAPS-buffered lysates from prostatic carcinoma tissues of intermediate and high Gleason grades (Gleason 5 and 9) were immunoprecipitated with anti-Bax and anti-Bak polyclonal antibodies and NRS as negative control. Immunoprecipitates were analyzed by Western blot using an anti-Bcl-xL monoclonal antibody.



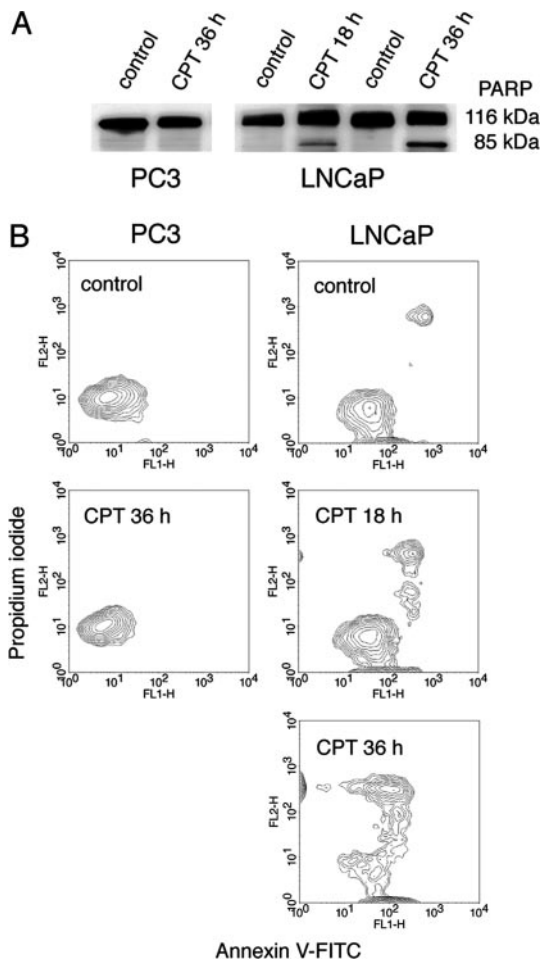


FIG. 3. Apoptosis induction by camptothecin in prostate cancer cells. A, Caspase-mediated cleavage of PARP was observed after 18 and 36 h of camptothecin (CPT) treatment in LNCaP but not PC3 cells. B, Camptothecin-induced apoptosis in LNCaP cells was also detected at 18 and 36 h as measured by flow cytometry. PC3 cells showed no changes, even 36 h after camptothecin treatment. FITC, Fluorescein isothiocyanate.

rosperine treatment, serum starvation, and p53 activation (40). We demonstrated that Bcl-xL is overexpressed in relation to prostate cancer progression.

We used two antibodies that recognize specifically the antiapoptotic Bcl-xL isoform to study a large series of patients by immunohistochemistry. We investigated the association of Bcl-xL overexpression with selected clinicopathological variables and the appearance of resistance to hormone therapy. We observed that Bcl-xL is significantly more abundant in high and intermediate Gleason-grade prostatic carcinomas than in more differentiated low-grade tumors. Bax and Bak were not differentially expressed in prostatic carcinomas of various Gleason grades, a fact that has been previously noted in the case of Bax (5). We also observed a statistically significant association with lymph node metastasis, distant metastasis, and initial serum PSA level. In the group of patients treated by complete androgen ablation, we found a significant association between the expression of Bcl-xL and the onset of hormone-refractory disease.

Actually, different models try to explain the activation

mechanism of multidomain proapoptotic proteins (30); however, it remains unclear how these proteins are maintained in an inactive state in the absence of any apoptotic stimulus. Willis *et al.* (17) demonstrated that Bak interacts with Bcl-xL and Mcl-1 in untreated HeLa cells; overexpressed Noxa binds Mcl-1 tightly and displaces Bak. The authors propose that other BH3-only proteins must be activated, provided that Bak-mediated apoptosis requires the neutralization of both Mcl-1 and Bcl-xL. Similarly, Shiao *et al.* (18) demonstrated recently that Bak interacts with Bcl-xL in untreated but not  $\alpha$ -tocopheryl succinate-treated PC3 cells. In these cases, Bak activation would be restrained by antiapoptotic Bcl-2 family members via direct interaction. In relation to Bax protein, several authors reported interactions of Bax with Bcl-xL (24, 25) and proteins unrelated to the Bcl-2 family (26–29) in untreated cells that kept Bax in an inactive state. Moreover, *in vitro* studies documented that multidomain proapoptotic proteins are executors of the mitochondrial pathway of apoptosis whose activation can be prevented by antiapoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-xL (41–44). However, whether this inhibition is via direct interaction between Bax and antiapoptotic proteins or not is still a matter of discussion because *in vivo* interactions seem to be dependent on the cellular model examined (23). The physiological significance of these interactions as well as the possible implication of the interactions status in untreated and apoptotic cells in the resistance or sensitivity to apoptosis remains to be fully understood.

Thus, we sought to investigate *in vivo* interactions of Bax and Bak with Bcl-xL in the prostate cancer cell lines LNCaP (androgen responsive) and PC3 (androgen unresponsive) by coimmunoprecipitation assays. We used CHAPS as the only detergent in the coimmunoprecipitation procedures, according to previous reports that demonstrate that immunoprecipitations with nonionic detergents may form artifactual complexes (45). We observed that both Bax and Bak coimmunoprecipitate with Bcl-xL in PC3 cells, but only Bak coimmunoprecipitates with Bcl-xL in LNCaP cells. In these cells, the interaction between Bax and Bcl-xL would occur to a much lesser extent, if any, than in PC3 cells. We also performed the immunoprecipitation with the anti-Bax antibody in the absence of any detergent and the interaction between Bax and Bcl-xL was again evidenced in PC3 but not LNCaP cells (data not shown). To assess whether these interactions occurred in primary prostate cancer, we also performed immunoprecipitation assays in prostatic carcinoma tissues of high and intermediate Gleason grades and found that Bcl-xL interacts with Bax and Bak only in the high-grade carcinoma. This finding supports that the described interactions may be of physiological relevance. As far as we know, this is the first time that *in vivo* interaction between Bax and Bcl-xL has been demonstrated in the prostate cancer PC3 cell line as well as in primary prostatic tissues.

To further investigate whether Bcl-xL represents a blockade over proapoptotic multidomain proteins that has to be removed for the effectiveness of the apoptotic process, we treated PC3 and LNCaP cells with the topoisomerase I inhibitor camptothecin. In camptothecin-treated LNCaP cells, the level of Bcl-xL protein diminished gradually; at 36 h, when the majority of cells were apoptotic, Bcl-xL protein was

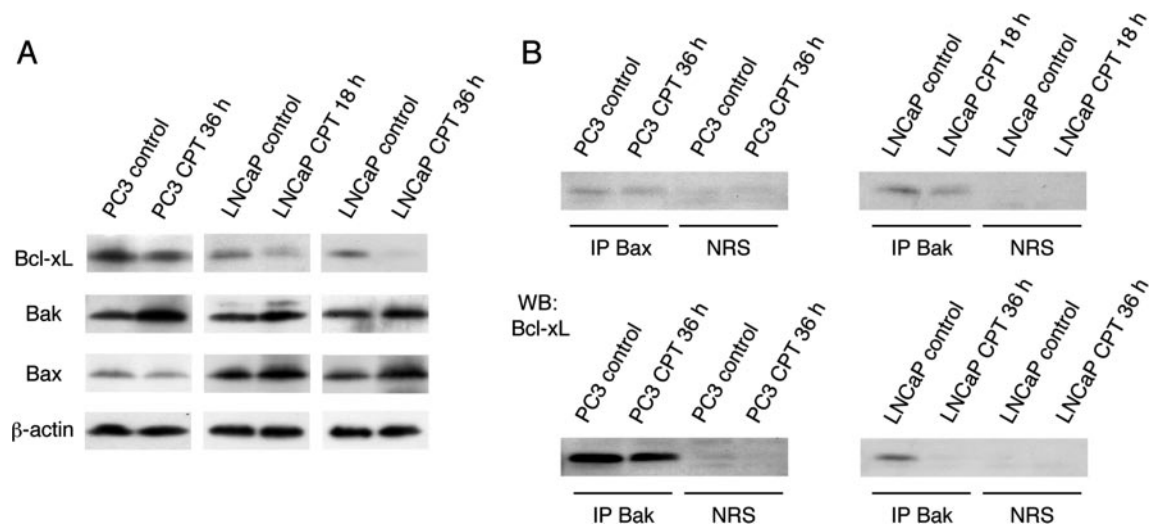


FIG. 4. Interactions of Bcl-xL with proapoptotic Bax and Bak in camptothecin-treated prostate cancer cells. A, Western blot analysis of Bax, Bak, and Bcl-xL in PC3 and LNCaP cells after camptothecin (CPT) treatment. B, Immunoprecipitation (IP) analysis of CHAPS-buffered lysates of camptothecin-treated PC3 and LNCaP cells using anti-Bax and anti-Bak polyclonal antibodies and normal rabbit serum (NRS) as negative control. Immunoprecipitates were analyzed by Western blot (WB) using an anti-Bcl-xL monoclonal antibody. In PC3 cells, interactions of Bcl-xL with Bax or Bak are not altered after camptothecin treatment. By contrast, the interaction between Bcl-xL and Bak in camptothecin-treated LNCaP cells is partially disrupted at 18 h and undetectable at 36 h.

almost undetectable, and thus, the interaction between Bcl-xL and Bak was absent. These findings support a model in which Bcl-xL would exert an inhibiting effect over Bak via heterodimerization. In unstimulated LNCaP cells, Bcl-xL would keep Bak in an inactive state; upon an apoptotic stimulus such as camptothecin treatment, it takes place a gradual loss of the interaction between these proteins that would allow Bak to exert its proapoptotic function. As previously reported (46), camptothecin did not induce apoptosis in PC3 cells, although it does inhibit cell growth, suggesting that the drug is reaching its target in the cell. Notably, the interactions between Bcl-xL with Bax and Bak were still present after 36 h of treatment. Thus, these observations suggest that the maintenance of the interactions between Bcl-xL with Bax and Bak in PC3 cells after apoptotic damage could be of relevance in the resistance to apoptosis, although the elucidation of this point would require further investigation. In this regard, the disappearance of the interaction between Bcl-xL and Bak during  $\alpha$ -tocopheryl succinate-induced apoptosis in PC3 cells supports this notion (18).

In this context, heterodimerization of Bcl-xL with Bax or Bak in high-grade prostatic carcinoma and camptothecin-treated PC3 cells may represent a mechanism of apoptosis blockade that facilitates tumor progression and androgen resistance, and that is absent in either carcinomas of moderate grade or LNCaP cells.

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