BCL-xL Is a Target Gene Regulated by Hypoxia-inducible Factor-1α*

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The transcription factor hypoxia-inducible factor-1α (HIF-1α) plays pivotal roles in physiology and pathophysiology. Constitutive or hypoxia-induced HIF-1α overexpression is observed in many types of cancers including prostate adenocarcinoma, in which it is associated with resistance to apoptosis and therapeutic agents. BCL-xL, a hypoxia-responsive, anti-apoptotic protein of the Bcl-2 family, is also overexpressed in prostate carcinoma and many other cancers. Despite this connection, whether BCL-xL expression is directly regulated by HIF-1α is not known. We used prostate cancer PC-3 cell with constitutive high HIF-1α level as a model to address this important question. We first generated prostate cancer PC-3 cells in which HIF-1α was stably knocked-down (HIF-KD) by using small interference RNA. BCL-xL was dramatically decreased in HIF-KD PC-3 cells, in parallel with sensitization to apoptosis with caspase-3 activation as well as decreased cell proliferation. We then demonstrated that HIF-1α directly regulated BCL-xL transcription by binding to a hypoxia-responsive element in the BCL-xL promoter (−865 to −847) by reporter gene assay, chromatin immunoprecipitation, and electrophoretic mobility shift and supershift assays. HIF-1α-dependent BCL-xL overexpression may be an important mechanism by which HIF-1α protects prostate cancer cells from apoptosis and leads to treatment resistance.

The transcription factor hypoxia-inducible factor-1α (HIF-1α) plays major roles in cellular response to hypoxia as well as in disease processes including carcinogenesis (1–4). Many genes have been identified as HIF-1 targets (3, 4), including GLUT-1, GAPDH, and VEGF, which are involved in such biological processes as energy metabolism, cell survival, and angiogenesis. Hypoxia inhibits proteasome-dependent degradation of HIF-1α, resulting in HIF-1α stabilization, which dimerizes with HIF-1β and activates target genes by binding to hypoxia responsive element (HRE) within their promoters.

Hypoxia and HIF-1α overexpression are implicated in the pathogenesis of many cancers, including prostate carcinoma (3–5), in which it is associated with advanced clinical stage and treatment failure (6). HIF-1α overexpression has been identified in both prostate adenocarcinoma tissue (5, 7) and cell lines (8).

Although acute hypoxia may lead to cell death, prolonged hypoxia results in resistance to apoptosis as well as to radiotherapy and chemotherapy (4, 9, 10), the mechanism of which is not well understood. Only recently have a few apoptosis regulators been identified as HIF-1α target genes, most notably the anti-apoptotic Mcl-1 (11) and BIRC5/survivin (12). Although pro-apoptotic molecules BNIP3, NIX (13, 14), and Noxa (15) are also responsive to HIF-1α, hypoxia-induced apoptosis-resistant phenotype eventually predominates.

BCL-xL (BCL2-like 1 or BCL2L1), a major anti-apoptotic protein of the Bcl-2 family, is also overexpressed in prostate carcinoma and many other cancers. BCL-xL overexpression is associated with the hormone-refractory phenotype and renders prostate cancer cells apoptosis-resistant, whereas BCL-xL knock-down increases sensitivity to chemotherapeutic agents (16, 17). Despite the correlation of BCL-xL overexpression with HIF-1α in some tumors (18) and the observation that BCL-xL is a key molecule underlying hypoxia-driven cell death resistance (10), the mechanism by which hypoxia induces BCL-xL expression is unclear, as it has not been elucidated if HIF-1α directly regulates BCL-xL.

BCL-xL gene is regulated by several transcription factor families, including STATs (signal transducers and activators of transcription) (19), NF-κB (20), Ets (21), GATA (22), PAX3 (and the PAX3/FKHR (Forkhead related transcription factor) fusion) (23), and POU (Brn-3a) (24). These regulators, however, are not closely related to hypoxia as HIF-1α.

We tested the hypothesis that BCL-xL is under HIF-1α regulation using prostate cancer PC-3 cell as a model, in which HIF-1α level is constitutively high. We show that stable knock-down of HIF-1α by small interference RNA (siRNA) results in a dramatic decrease of BCL-xL with consequent increase in

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§The abbreviations used are: HIF-1α, hypoxia-inducible factor-1α; BCL-xL, BCL2L1 or BCL2-like 1; BNIP3, BCL2/adenosine E1B 19-kDa interacting protein 3; CASP, caspase; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT-1, glucose transporter-1; HIF-KD, HIF-1α knock-down PC-3 cells; HRE, hypoxia-responsive element; IAP, inhibitor of apoptosis protein; Mcl-1, myeloid cell factor-1; MITT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NIX, BCL2/adenosine E1B 19-kDa interacting protein 3-like; RNAi, RNA interference; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP digoxigenin nick end labeling; VEGF, vascular endothelial growth factor; PI3K, phosphatidylinositol 3-kinase; RT, reverse transcription; KD, knocked-down; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; CMV, cytomegalovirus.
apoptosis, and most importantly, BCL-xL is transcriptionally regulated by HIF-1α.

EXPERIMENTAL PROCEDURES

Cells, Tissues, and General Reagents—Human prostate cancer cell lines LNCaP, DU145, and PC-3 were maintained in RPMI1640 with 10% fetal calf serum (Invitrogen). Prostate adenocarcinoma tissue and normal prostate tissue (from prostatectomy specimens of non-prostate diseases) were snap-frozen in accordance with institutional guidelines. Normal prostate epithelial cells were collected by laser capture microdissection with the Leica AS LMD system (Leica Microsystems, Wetzler, Germany). The phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 was from Sigma. Tris base, Tween 20, dithiothreitol, and EDTA were from Amresco (Solon, OH). Phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and aprotonin were from Roche Diagnostics.

Reverse Transcription (RT)-PCR and Real-time Quantitative PCR—Total RNA was extracted by using the Trizol reagent (Invitrogen). Reverta Ace reverse transcriptase (ToYoBo, Osaka, Japan) was used for RT. PCR primers were designed according to cDNA sequences (GenBank™) as follows: HIF-1α (5’-CCT ATG ACC TGC TTG GTG CTG-3’, 5’-CTG GCT CCA CCC ATG-3’, product length 157 bp), BNIp3 (5’-ACC AAC AGG GCT TGC TTA GTC-3’, 5’-GAG GGT GGC TGC C-3’, 202 bp), GLUT-1 (5’-GCA AGT CCT TTG AGA TGC TGA TTC-3’, 5’-GCG GAC TCT TGC TCT-3’, 402 bp), GAPDH (5’-CCA ACC ATG GCA AAT TCC ATG GCA-3’, 5’-TCA AGA CGG CAG GTG-3’, 597 bp), BCL-xL/BCL-xS (5’-CTG GCT CCT GTG CAC-3’, 244 bp), BIRC5/survivin (5’-CTG TTC AGC TGC TCT GCT-3’, 200 bp), HIF-1α control plasmids (RNAi-CON1 and RNAi-CON2, respectively) and scrambled sequences were also designed and prepared as Scrambled 1, 379–397). Corresponding control siRNAs with scrambled sequences were also designed and prepared as Scrambled 1, GACCTACACTACCTATCA, and Scrambled 2, GTGGA-CACCAGTTT. These sequences were checked to ensure non-homology with known human mRNA sequences. Co-expression of green fluorescence protein from the plasmid was used for checking transfection efficiency.

PC-3 cells were transfected by using Lipofectamine 2000 (Invitrogen). To obtain HIF knock-down cells (HIF-KD) with stable transfection of HIF-1α siRNA1 and HIF-1α-siRNA2 (designated as HIF-KD1 and HIF-KD2, respectively) and the control plasmids (RNAi-CON1 and RNAi-CON2, respectively), cells were selected by G418 at 500 μg/ml for 2 weeks (starting at 48h after transfection) and maintained in growth medium supplemented with G418 (200 μg/ml).

Western Blot Analysis—The primary antibodies used were: HIF-1α (mouse monoclonal, 1:500) from Chemicon Inc., Pittsburgh, PA; BCL-xL (rabbit polyclonal, 1:1,000) and phosphorylated AKT (rabbit polyclonal, 1:600) from Cell Signaling Technology Inc., Danvers, MA; BNIp3 (mouse monoclonal, 1:3,000) from Sigma; BIRC5/survivin (rabbit polyclonal, 1:1,000) from R and D Systems Inc., Minneapolis, MN; BAX (mouse monoclonal, 1:800), CIAP1 (rabbit polyclonal, 1:800), CIAP2 (rabbit polyclonal, 1:800), CASP3 (rabbit polyclonal, 1:800), CASP9 (rabbit polyclonal, 1:600), and AKT1/2 (goat polyclonal, 1:800) from Santa Cruz Biotechnology, Santa Cruz, CA; GAPDH (mouse monoclonal, 1:10,000) from Kangcheng, Shanghai, China; and β-tubulin (mouse monoclonal, 1:1,000) from Huatesheng, Shenzhen, China. Horseradish peroxidase-labeled secondary antibodies were from Zymed Laboratories Inc.

The Real-time PCR Master Mix containing SYBR Green (ToYoBo) was used for real-time PCR on Light Cycler 2.0 (Roche Diagnostics), and data were recorded and analyzed by the Light Cycler software 4.05. Copy number of target genes (relative to β-actin) was defined by 2^(-ΔΔCt), where ΔΔCt = ΔCt_HIF-KD - ΔCt_HIF-CON = (Ct_HIF-KD-target - Ct_HIF-KD-actin) - (Ct_HIF-CON-target - Ct_HIF-CON-actin).

Inhibition of PI3K in PC-3 Cells—PC-3 cells were cultured in 6-well plates in fetal calf serum-free media and treated with 0, 20, and 50 μM PI3K inhibitor LY294002 for 1 h. Cells were collected for Western analysis of HIF-1α, BCL-xL, AKT1/2, and phosphorylated AKT.

Hypoxia Mimetic Treatment of PC-3 Cells—PC-3 cells were cultured in 6-well plates in fetal calf serum-free media and treated with 0, 200, 400 μM CoCl2 for 4 h. Cells were collected for Western analysis of HIF-1α and BCL-xL.

HIF-1α RNA Interference—The expression vector pRNAT-U6.1Neo (GenScript Corp, Piscataway, NJ) was used to construct HIF-1α siRNA plasmids by inserting siRNA-coding sequences under U6 promoter for siRNA expression. Two (HIF-siRNA1 and -2) expressing vectors were constructed with the following siRNA sequences: HIF-siRNA1, GCCACATCATTCCATATA (nucleotides 1960–1978, NM_001530); HIF-siRNA2, CTAATGACAGACTGTGT (nucleotides 379–397). Corresponding control siRNAs with scrambled sequences were also designed and prepared as Scrambled 1, GCCATCAACATTACCTAGCA, and Scrambled 2, GTGGACACCGATAAGTTT. These sequences were checked to ensure non-homology with known human mRNA sequences.

Total proteins resolved by SDS-polyacrylamide (Sigma) gel electrophoresis were electroblotted to polyvinylidene difluoride membrane (Amersham Biosciences), blocked with 5%...
BCL-xL Regulation by HIF-1α

nonfat milk and 0.1% Tween 20, and incubated with primary and secondary antibodies at room temperature for 2 and 1.5 h, respectively. Signals were detected by exposure to x-ray films after treatment with the SuperSignal enhanced chemiluminescence kit (Pierce).

Ultraviolet (UV) Irradiation Induced Cell Death—Cells in culture plates were briefly exposed to UV irradiation in a UV cross-linker (UVC-500, Hoefer, San Francisco, CA) at 120 ml/cm² for 30 s. Cells were then cultured as appropriate for subsequent assays.

Immunocytochemistry—Cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 and incubated overnight at 4°C with anti-human Ki67/MIB-1 antibody (mouse monoclonal, 1:100, DakoCytomation, Glostrup, Denmark), anti-human cleaved caspase-3 antibody (rabbit polyclonal, 1:200, Cell Signaling Technology), or anti-human BCL-xL antibody (1:200). Standard labeled streptavidin-biotin protocol was used for staining with 3’-diaminobenzidine as chromogen and hematoxylin as counterstain.

Cell Viability Assay—Cells were cultured in 96-well plates and measured by tetrazolium-based MTT (Sigma) cell proliferation assay. The working concentration of MTT was 1 mg/ml.

Caspase-3 Activity Assay—Cultured cells were lysed with lysis buffer containing 50 mM Hepes (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, and 10 mM dithiothreitol. The soluble fraction of the cell lysate was used for colorimetric caspase-3 activity assay using acetyl-Asp-Glu-Val-Asp-\(\text{-nicotinamide} \) (Ac-DEVD-PNA) (Calbiochem) as a substrate on FL600 plate reader (BIO-TEK, Winooski, VT).

Terminal Deoxynucleotidyltransferase-mediated Biotinylated \(dUTP \) Nick end-labeling (TUNEL)—TUNEL was performed by using in situ cell death detection kit (Roche Diagnostics). Cells were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton-100, 0.1% sodium citrate, incubated with TUNEL reaction mixture then with alkaline phosphatase-conjugated anti-fluorescein antibody, stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate and counterstained with methyl green. Reaction without terminal transferase was used as negative control. The Apoptotic index was represented as number of TUNEL(+) cells/total number of cells (%).

Overexpression of BCL-xL in HIF-KD1 Cells—Full-length cDNA of BCL-xL coding sequence was cloned into TA vector pMD18-T (TaKaRa, Dalian, China) and subcloned into pDsRed vector (Clontech, Palo Alto, CA). The primers used for cloning were: 5’-AGA TCT AAT GTC TCA GAG CAA CCG GGA-3’ and 5’-GTC GAC CGT TTC CGA CTG AAG AGT GAG-3’.

HIF-KD1 cells were transfected with BCL-xL expression plasmid (HIF-KD1-xL) or pDsRed control vector (HIF-KD1) using Lipofectamine 2000. Transfected HIF-KD1-xL and HIF-KD1-DsRed cells were maintained in RPMI1640.

Reporter Gene Assay for HIF-1α-dependent BCL-xL Promoter Activity—The basic pGL3 luciferase reporter vector (Promega, Madison, WI) was used to construct reporter plasmids with various lengths of the BCL-xL promoter. Four plasmids were constructed in which the BCL-xL promoter spanned -1075 to +617 (relative to the transcription start site) or truncated fragments of which were inserted upstream of the luciferase gene. The reporter constructs were designated as pGL1642 (-1075 to +617), pGL1281 (-664 to +617), pGL828 (-211 to +617), and pGL621 (-4 to +617), respectively. Two additional plasmids with HRE1 and HRE2 site-specific mutation were constructed: pGL828-MUT (-211 to +617, with CGTG at -78 to -75 of HRE1 mutated to TCGG) and pGL1642-MUT (-1075 to +617, with CGTG at -858 to -855 of HRE2 mutated to TCGG). Each reporter construct and the pRL-CMV plasmid (Promega) containing the Renilla luciferase gene as internal control were used in dual reporter gene assay for studying HIF-1α-dependent gene expression. Cells were transfected with plasmids by using Lipofectamine 2000 (Invitrogen). Four hours after transfection, the medium was replaced by fresh medium. Thirty-six hours after transfection, cells were treated with 400 \( \mu \)M CoCl₂ for 12 h, and luciferase activity was determined by using Luminometer TD-20/20 (Turner Designs, Sunnyvale, CA).

Chromatin Immunoprecipitation—Cells were lysed, and nuclei were pelleted. The extract was sonicated, and supernatants were collected and treated with sheared salmon sperm DNA (Invitrogen) and protein A/G-Sepharose (Santa Cruz). Immunoprecipitation was performed overnight at 4°C with 3 \( \mu \)g of HIF-1α monoclonal antibody or the control isotype IgG2b (Lab Vision Corp., Fremont, CA) or no antibody and then with protein A/G-Sepharose and salmon sperm DNA. Precipitates were washed, and extracted with 1% SDS and 0.1 M NaHCO₃. Eluates were pooled and heated. DNA fragments were purified and used as template for PCR. The promoter-specific primers used were: BCL-xL, 5’-CGA GCA GTC AGC CAG GTA G-3’ and 5’-GAC GGC GAA GGC TCC TAT TG-3’; VEGF (as positive control), 5’-GTT CCC TGG CAA CAT CTG G-3’ and 5’-GAC ATC AAA GTG AGC GCC AG-3’.

Electrophoretic Mobility Shift Assay (EMSA) and Supershift Assay—The sequences of the two BCL-xL promoter oligonucleotide probes were: BCL-xL-Pro1, 5’-GAGCCAAGGGGCGTGAAGAGAGAGG-3’ (-89 to -64), and BCL-xL-Pro2, 5’-CCCTGTGCGTCCTCACCAG-3’ (-865 to -847). The VEGF promoter probe 5’-GATGACACGCTGGGCTTCCA-3’ (-989 to -970) was used as positive control. Three corresponding probes with HRE mutation were prepared: BCL-xL-Pro1-MUT, 5’-GAGCCAAGGGGCCGTCGGAAGAGAGAGG-3’ and BCL-xL-Pro2-MUT, 5’-CCCTGTGCGTCCTCACCAGGCTTCCA-3’, VEGF-Pro-MUT, 5’-GATGACATCATCGGCTGGGCTTCCA-3’. Labeled wild-type probes were prepared by biotinylation (Invitrogen). Unlabeled wild-type and mutant probes were used for competition experiments. For each probe, complementary strands were synthesized, and equimolar concentrations of complementary strands were annealed for use in EMSA.

PC-3 cells were harvested after 6 h of incubation with 400 \( \mu \)M CoCl₂. Nuclear extract was prepared, and 10 \( \mu \)g was incubated with 100 pmol of biotinylated probe in a 10-\( \mu \)L reaction mixture (with 0.5 \( \mu \)g of poly(dI-dC)) for 30 min at room temperature. For competition assays, a 50-fold excess of unlabeled wild-type probe or mutant probe was used. For supershift assays, 1.0 \( \mu \)g of monoclonal anti-HIF-1α antibody was added to the reaction mixture and incubated at 4°C overnight. The mixture was electrophoresed at 4°C on 6% PAGE for 3 h and transferred to
nylon membranes (Roche Diagnostics) by electroblotting. After baking and blocking, horseradish peroxidase-labeled streptavidin (1:1000, Zymed Laboratories Inc.) was added and incubated at room temperature for 2 h. Signals were detected by exposure to x-ray films after treatment with the SuperSignal enhanced chemiluminescence kit (Pierce).

Statistical Analysis—Statistical analysis was performed by using the SPSS 10 software package (Chicago, IL).

RESULTS

HIF-1α siRNA Significantly Decreased Expression of HIF-1α and Its Target Genes—HIF-1α mRNA and protein overexpression in prostate cancer cells and primary prostate adenocarcinoma tissues was validated by conventional RT-PCR (Fig. 1A) and Western blot (Fig. 1B) analysis, respectively. The two HIF-1α siRNA constructs (HIF-1α-siRNA1 and 2) significantly knocked down HIF-1α level, as measured by conventional (C) or real-time quantitative RT-PCR (D) and Western blot analysis (E). The respective scrambled siRNA (Scrambled 1 and 2) had no effects. G418-selected stable HIF-1α knockdown cells (HIF-KD1 and HIF-KD2) and control cells (RNAi-CON1 and RNAi-CON2) were homogeneously green fluorescence protein-expressing (F). Knock-down of HIF-1α resulted in significant down-regulation of HIF-1α target genes BNIP3, GLUT-1, and GAPDH in HIF-KD cells in comparison to RNAi-CON cells, as shown by RT-PCR (G) and Western blot analysis (H).

HIF-1α siRNA Inhibited PC-3 Cell Proliferation—HIF-KD cells also showed significantly reduced cell growth (Fig. 2, A and B). In contrast, the control constructs with scrambled sequences had no effect on HIF-1α expression. The G418-selected PC-3 cells with stably transfected HIF-siRNA1 and HIF-siRNA2 (HIF-KD1 and HIF-KD2 cells, respectively) and the control plasmids (RNAi-CON1 and RNAi-CON2 cells, respectively) were largely homogeneous, as shown by the co-expression of green fluorescence protein (Fig. 1F).

HIF-1α mRNA (Fig. 1G) and protein (Fig. 1H) were significantly reduced in the HIF-KD cells, with consequent down-regulation of known HIF-1α target genes BNIP3, GLUT-1, and GAPDH (Fig. 1, G and H). The control constructs had no effect on mRNA and protein expression of HIF-1α or its target genes (Fig. 1, G and H). Quantitative PCR analysis of mRNA of HIF-1α and its target gene GLUT-1 further validated the interference effect of HIF-1α siRNA, as both of which were significantly reduced (not shown).

BCL-xL Regulation by HIF-1α

FIGURE 1. HIF-1α siRNA significantly decreased HIF-1α overexpression in prostate cancer cells. HIF-1α mRNA (A) and protein (B) were overexpressed in prostate cancer cells PC-3, LNCaP, and DU-145, as were in primary prostate cancer tissue (PCa) but were undetectable in normal prostate epithelium (NP) collected by laser capture microdissection. β-Actin and tubulin were used as control for the RT-PCR and Western blot analysis, respectively. The two HIF-1α siRNA constructs (HIF-1α-siRNA1 and 2) significantly knocked down HIF-1α level, as measured by conventional (C) or real-time quantitative RT-PCR (D) and Western blot analysis (E). The respective scrambled siRNA (Scrambled 1 and 2) had no effects. G418-selected stable HIF-1α knockdown cells (HIF-KD1 and HIF-KD2) and control cells (RNAi-CON1 and RNAi-CON2) were homogeneously green fluorescence protein-expressing (F). Knock-down of HIF-1α resulted in significant down-regulation of HIF-1α target genes BNIP3, GLUT-1, and GAPDH in HIF-KD cells in comparison to RNAi-CON cells, as shown by RT-PCR (G) and Western blot analysis (H).
Decreased cell proliferation was further demonstrated by immunocytochemistry of the proliferative antigen Ki67/MIB-1, which showed much lower Ki67-labeling index (50%) in HIF-KD cells than control (90%) (Fig. 2A). The dark brown-stained nuclei were Ki67/MIB-1-positive proliferating cells. MTT assay further showed the decreased cell growth of HIF-KD as compared with RNAi-CON cells (mean ± S.D. of three independent experiments, \( p < 0.01 \) ) (B). HIF-1α siRNA also sensitized HIF-KD cells to anti-androgen drug flutamide \( (10^{-6} \text{ M}) \), which further inhibited cell growth of HIF-KD cells but was ineffective on RNAi-CON cells (B). HIF-KD cells were rendered more sensitive to UV irradiation-induced cell death (C). TUNEL assays demonstrated significant increase of apoptotic index \((A)\) upon UV-irradiation in HIF-KD cells but not in RNAi-CON cells (mean ± S.D. of three independent experiments, \( p < 0.01 \) ) (D), concomitant with increased levels of cleaved caspase-3 \((E)\), immunocytochemistry, cytoplasmic brown-staining, as a manifestation of caspase-3 activation) and increased caspase-3 activity \((F)\), colorimetric assay of cell extract, mean ± S.D. of three independent experiments, \( p < 0.01 \) ).

**BCL-xL Regulation by HIF-1α**

HIF-KD Cells Were More Sensitive to Ultraviolet Irradiation and Flutamide Treatment—HIF-KD cells showed a significantly higher cell death rate (Fig. 2, C and D) after UV irradiation. TUNEL assays demonstrating the spontaneous apoptotic decrease in expression upon HIF-1α siRNA. As the base level of BCL-xL was extremely low, the effect appeared less dramatic than BCL-xL (Fig. 3A).

As expected, HIF-1α siRNA also induced a significant decrease of survivin, a documented HIF-1α target (Fig. 3, A–D). Other members of the IAP family, including CIAP1, CIAP2, and XIAP, were not down-regulated. Moreover, HIF-1α siRNA had no effect on expression level of the examined caspases (Fig. 3, A and B).
Inhibition of the PI3K/Akt Pathway Resulted in Down-regulation of Both HIF-1α and BCL-xL—The down-regulation of BCL-xL by HIF-1α siRNA was dramatic given the constitutive high BCL-xL mRNA (Fig. 5A) and protein (Fig. 5B) level in prostate cancer cell lines. Because PI3K/Akt is a major signaling pathway that controls HIF-1α level, we tested if PI3K inhibitor LY294002 could lead to decrease of BCL-xL. As shown in Fig. 5C, both HIF-1α and BCL-xL were simultaneously reduced upon PI3K/Akt inhibition in a dose-dependent manner.

Hypoxia Mimetic CoCl2 Boosted Concomitant BCL-xL and HIF-1α Expression in PC-3 Cells—Treatment of PC-3 cells with the hypoxia mimetic CoCl2 resulted in additional increase of BCL-xL and HIF-1α simultaneously, further supporting the potential dependence of BCL-xL on HIF-1α in response to hypoxia (Fig. 5D).

Potential HREs on BCL-xL Promoter—The above experiments provided important clues to relationship between HIF-1α and BCL-xL. Because BCL-xL is a gene regulated by NF-κB, which could potentially be activated by PI3K/Akt signaling, reduction of BCL-xL expression by PI3K/Akt inhibition might be the effect of either HIF-1α or NF-κB inhibition or both. This prompted us to investigate whether BCL-xL was directly regulated by HIF-1α.

Potential HRE was searched in the human BCL-xL promoter region. Four short HRE consensus motifs were identified within the ~1000-bp region preceding the transcriptional start site. Two of them, starting at positions −78 and −858 (Fig. 6A), fit the extended consensus and were highly conserved across species (Fig. 6B).

Overexpression of BCL-xL in HIF-KD Cells Promoted Cell Growth and Inhibited Cell Death—To further demonstrate the importance of HIF-induced BCL-xL expression in apoptosis resistance, BCL-xL was artificially overexpressed in HIF-KD cells (Fig. 4, A–E), which counteracted the effects of HIF-1α siRNA, resulting in enhanced cell growth (Fig. 4C) and inhibition of UV irradiation-induced cell death and caspase 3 activity (Fig. 4, D and E).

Reporter Gene Assay of Putative HRE Activity—The reporter constructs were shown in Fig. 6C. The pGL1642 (−1075 to +617) contained both HREs (HRE2 and HRE1) flanking two NF-κB binding sites, whereas pGL1281 (−664 to +617) differed by lacking HRE2. The pGL828 (−211 to +617) contained HRE1 only, and pGL621 (−4 to +617) contained no HRE. Two constructs were prepared with site-specific mutation of the respective HREs: pGL828-MUT and pGL1642-MUT.
PC-3 cells were transiently transfected with one of the six constructs, with PRL-CMV co-transfection as internal control. The hypoxia mimetic CoCl$_2$ was used to simulate hypoxia and to further boost HIF-1$\alpha$ as a means of showing hypoxia-induced gene transcription in the reporter gene assay.

The experiment showed significantly higher luciferase activity of the HRE-bearing constructs (Fig. 6C) than base line (pGL3). The effects were more dramatic with the HRE2-bearing construct upon CoCl$_2$ treatment, whereas mutation of the HRE (notably HRE2) core sequence resulted in a significant reduction of transcriptional activity. These experiments indicated that the reporter gene transcription was under control of BCL-xL promoter containing HRE, particularly HRE2, which responded to the hypoxia mimic.

Chromatin Immunoprecipitation Assay Displayed HIF-1$\alpha$ Interaction with BCL-xL Promoter—To show HIF-1$\alpha$ physically bind to BCL-xL promoter, we first used chromatin immunoprecipitation assay of PC-3 cells treated by CoCl$_2$. Using the chromatin fraction pulled down by anti-HIF-1$\alpha$ antibody as template, a PCR fragment corresponding to −1025 to −821 (containing HRE2) of BCL-xL promoter was detected (Fig. 6D) and verified by sequencing. This fragment was not detected when isotype control IgG2b or no antibody was used for the pulldown assay (Fig. 6D).

EMSA and Super Shift Assay Demonstrated HIF-1$\alpha$ Binding to the Promoter Region of BCL-xL—To further confirm binding of HIF-1$\alpha$ to the putative HRE in human BCL-xL promoter, we performed EMSA with two oligonucleotide probes, each containing one of the extended HRE consensus sequence, designated BCL-xL-Pro1 (−89 to −64) and BCL-xL-Pro2 (−865 to −847), respectively. Two probes with HRE core sequence mutations were also prepared for competition experiments and were designated as BCL-xL-Pro1-MUT and BCL-xL-Pro2-MUT, respectively. A known HIF-1$\alpha$ binding oligonucleotide derived from the VEGF receptor gene promoter (VEGF-Pro) was used as positive control (Fig. 6E) together with a corresponding HRE mutation probe, VEGF-Pro-MUT, for competition assays.

DISCUSSION

We generated prostate cancer PC-3 cells in which HIF-1$\alpha$ was stably knocked-down by using siRNAs, which resulted in a significant decrease of the anti-apoptotic molecule BCL-xL. We then showed that HIF-1$\alpha$ directly regulated BCL-xL gene transcription. These novel findings point to HIF-1$\alpha$-dependent BCL-xL overexpression as an important mechanism by which HIF-1$\alpha$ protects prostate cancer cells from apoptosis and leads to treatment failure.

Hypoxia is common in solid tumors (4), including prostate carcinoma (3), in which the extent of hypoxia is correlated with clinical stage and treatment failure (6). A hypoxia-mediated increase in HIF-1$\alpha$ plays critical roles in tumorigenesis and progression of many cancers through HIF-1$\alpha$-dependent activation of genes that promote cancer cell survival, proliferation, spreading, and angiogenesis. Overexpression of HIF-1$\alpha$ and its target genes has been observed in a variety of solid tumors, for example, tumors of the brain (25), kidney and the urinary tract (26), and lung (27) as well as prostate (5, 7).

High levels of HIF-1$\alpha$ have been observed in prostate cancer tissue and cell lines (5, 7, 8). Up-regulation of HIF-1$\alpha$ might be an early event in prostate carcinogenesis, as high grade prostate intraepithelial neoplasia showed higher a HIF-1$\alpha$ level than benign epithelium (5). It is noteworthy that, although HIF-1$\alpha$ overexpression is often hypoxia-dependent, prostate cancer cells have constitutively high HIF-1$\alpha$ level, which could be further increased by hypoxia (8). HIF-1$\alpha$ gene amplification (28) and P582S polymorphism or mutation in the oxygen-dependent domain (29) might contribute to overexpression of HIF-1$\alpha$ at normoxic conditions.

Hypoxia and HIF-1$\alpha$ overexpression contribute to resistance to radiotherapy and chemotherapy (4). For example, the multi-drug resistance 1 gene has been observed to be hypoxia-respon-
sive and is regulated by HIF-1α (30). Treatment of LNCaP cells with the androgen receptor antagonist Casodex results in up-regulation of a subset of hypoxia-related genes, including membrane metallo-endopeptidase and cyclin G2, which might be involved in development of the androgen-independent phenotype (31).

 Knock-down of HIF-1α by siRNA or antisense techniques inhibits cell growth, proliferation, or migration, and promotes apoptosis. The effects have been observed in human respiratory epithelium (32) and umbilical vascular endothelial cells (33) as well as in a variety of tumors, including glioma (34), non-small cell lung cancer (35), hepatocellular carcinoma (11), pancreatic cancer (36), pituitary adenoma (37), squamous cell carcinoma (38), and prostate cancer (39).

 Of more clinical interest is that silencing of HIF-1α gene results in sensitization of cancer cells to therapeutic agents. For example, HIF-1α knock-down increases sensitivity to 5-fluorouracil, doxorubicin, and gemcitabine in pancreatic cancer cell (36). Our finding that HIF-1α knock-down renders the androgen-independent PC-3 cells more sensitive to UV or flutamide treatment also supports that HIF-1α is a potential therapeutic target in androgen-independent prostate cancer.

The effects of such inhibition are mainly mediated by down-regulation of HIF transcriptional targets involved in diverse biological processes as cell proliferation, metabolism, and angiogenesis. These targets include, for example, phosphoglycerate kinase (35), GLUT-1 (34), chemokine receptors CXCR1 and CXCR2 (39) or CXCR4 (40), and VEGF (35).

Promotion of apoptosis is a recurrent theme of HIF-1α knock-down (11, 32–40). However, the underlying mechanisms have been less clear. Caspases could be increased or activated upon HIF-1α siRNA, but it most probably reflects the activation of caspase-dependent pathways rather than transactiva-

**FIGURE 6.** HRE in BCL-xL promoter; binding to and transcriptional regulation by HIF-1α. Two potential HRE sites (Site 1 and Site 2, starting at position −78 and −858 of human BCL-xL promoter, respectively) identified by sequence analysis (A) were conserved across species (B). Dual reporter gene assays were performed with expression constructs carrying various lengths of BCL-xL promoter (range relative to transcription start site) (C). The promoter regions were inserted into respective reporter gene (firefly luciferase) constructs, and the upper panel shows restriction analysis of the inserts (which have also been verified by sequencing). pGL1642 (−1075 to +617) contained HRE2 and HRE1 flanking two NF-x binding sites. pGL1281 (−664 to +617) lacked HRE2. pGL828 (−211 to +617) contained HRE1 only, and pGL621 (−4 to +617) contained no HRE. In pGL642-MUT and pGL828-MUT, the respective HRE was mutated. PC-3 cells were co-transfected with one of the six constructs together with pRL-CMV (which carried the Renilla luciferase gene controlled by CMV promoter, as internal control). CoCl2 was used to simulate hypoxia and to boost HIF-1α level. The reporter gene activity, represented by relative luciferase activity (firefly/Renilla), was significantly increased over the base line when HRE2 was present, and the cells were stimulated with CoCl2 (C). In contrast, pGL642-MUT significantly reduced the reporter gene activity. Constructs with HRE1 only (pGL828-MUT) had little effect, indicating HRE1 was not effectively regulated by HIF-1α. Also notice that the presence of the NF-x binding site resulted in significantly higher reporter gene activity over constructs lacking NF-x binding site, indicating significant contribution of this binding site to BCL-xL promoter activity. The chromatin immunoprecipitation assay was used to show HIF-1α binding to the BCL-xL promoter (D). PCR using chromatin (Input) pulled down by anti-HIF-1α antibody (HIF-Ab) as template yielded the BCL-xL-Pro fragment (−1023 to −821 containing HRE2) of the BCL-xL promoter, which was verified by sequencing. When isotype control IgG2b or no antibody (No Ab) was used for the pulldown assay, no PCR product was observed. PCR of VEGF promoter (VEGF-Pro) was used as positive control. EMSA was performed to further confirm HIF-1α binding to HRE (E). Oligonucleotide probes BCL-xL-Pro1 (−89 to −64) and BCL-xL-Pro2 (−865 to −847) contained HRE1 and HRE2, respectively. The HRE core sequence was mutated in the corresponding BCL-xL-Pro1-MUT and BCL-xL-Pro2-MUT probes. A known HIF-1α binding oligonucleotide derived from the VEGF receptor gene promoter (VEGF-Pro) was used as positive control together with a corresponding HRE mutation probe, VEGF-Pro-MUT. The biotin-labeled BCL-xL-Pro2 (E, lanes 1 and 2), but not BCL-xL-Pro1 (not shown), caused gel mobility shift when incubated with nuclear proteins from PC-3 cells. The shift was suppressed by competition with excess unlabeled wild-type probe BCL-xL-Pro2 (E, lane 3) but not with the HRE-mutated probe BCL-xL-Pro2-MUT (E, lane 4). When HIF-1α monoclonal antibody (HIF-Ab) was included in the binding reaction, a supershift band was observed with BCL-xL-Pro2 (E, lane 5) but not BCL-xL-Pro1 (not shown). Gel mobility shift and supershift assays were also shown with the VEGF-Pro positive control (E, lanes 6 and 7) together with competition and supershift assays (E, lanes 8–10).
BCL-xL Regulation by HIF-1α

It was only recently that two anti-apoptotic genes, BIRC5/survivin (12) and McI-1 (11, 32), were identified as HIF-1α targets. Despite responsiveness to HIF-1α by pro-apoptotic genes BNIP3, NIX, and Noxa (13–15), most experiments have shown that knock-down of HIF-1α promotes cell death and inhibits cell proliferation (32–35, 37, 38), apparently as the end result of a complex regulatory circuit.

Although BCL-xL has been found to be a key molecule involved in hypoxia-induced resistance to cell death (10) and BCL-xL overexpression has been associated with increased HIF-1α in tumors such as non-small cell lung cancer (18), the mechanism by which hypoxia induces BCL-xL up-regulation and the relationship between HIF-1α and BCL-xL has not been known.

Our study, thus, provides the first evidence that HIF-1α directly regulates BCL-xL transcription by interacting with HRE in the BCL-xL promoter. It has been shown that in the androgen-independent PC-3 cell, BCL-xL is more responsible for apoptosis-resistance than the prototypic Bcl-2 (16). Our data, therefore, indicate that HIF-1α-dependent overexpression of BCL-xL in PC-3 cells is one of the major mechanisms by which prostate cancer cells, particularly androgen-independent cells, resist apoptosis and chemotherapy. Recently, the IAP family member survivin has been identified as a transcriptional target of HIF-1α (12). Being up-regulated in many cancers, survivin is reported to be involved in the regulation of both apoptosis and cell division. Thus, HIF-1α overexpression (either constitutive or hypoxia-induced) may promote tumorigenesis by exerting direct effects on key members of major gene families controlling cell death and proliferation; that is, the inhibition of cell death by up-regulating BCL-xL and survivin and promotion of cell proliferation by up-regulation of survivin. Elucidation of HIF-1α-dependent BCL-xL expression may provide a new dimension for understanding BCL-xL regulation.

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