Alleviating the Suppression of Glycogen Synthase Kinase-3β by Akt Leads to the Phosphorylation of cAMP-response Element-binding Protein and Its Transactivation in Intact Cell Nuclei*

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Glycogen synthase kinase-3β (GSK-3β) activity is suppressed when it becomes phosphorylated on serine 9 by protein kinase B (Akt). To determine how GSK-3β activity opposes Akt function we used various methods to alleviate GSK-3β suppression in prostate carcinoma cells. In some experiments, LY294002, a specific inhibitor of phosphatidylinositol 3-kinase (a kinase involved in activating Akt) and tumor necrosis factor-α (TNF-α) were used to activate GSK-3β. In other experiments mutant forms of GSK-3β, GSK-3βY216F (a constitutively active deletion mutant of GSK-3β) and GSK-3βT181N/F (an inactive point mutant of GSK-3β) were used to alter GSK-3β activity. LY294002, TNF-α, and overexpression of wild-type GSK-3β or of GSK-3β39, but not GSK-3β39, alleviated the suppression of GSK-3β activity in prostate carcinoma cells and enhanced the turnover of β-catenin. Forced expression of wild-type GSK-3β or of GSK-3β39, but not GSK-3β39, suppressed cell growth and showed that the phosphorylation status of GSK-3β can affect its intracellular distribution. When transcription factors activator protein-1 and cyclic AMP-response element (CRE)-binding protein were analyzed as targets of GSK-3β activity, overexpression of wild-type GSK-3β suppressed AP1-mediated transcription and activated CRE-mediated transcription. Overexpression of GSK-3β39 caused an (80-fold) increase in CRE-mediated transcription, which was further amplified (up to 130-fold) by combining GSK-3β39 overexpression with the suppression of Jun activity. This study also demonstrated for the first time that expression of constitutively active GSK-3β39 results in the phosphorylation of CRE-binding protein on serine 129 and enhancement of CRE-mediated transcription in intact cell nuclei.

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† The abbreviations used are: GSK-3β, glycogen synthase kinase-3β; Akt, protein kinase B; CRE, cAMP response element; CBP, CREB binding protein; AP1, activator protein-1; P13K, phosphatidylinositol 3-kinase; IGF, insulin-like growth factor; TNF, tumor necrosis factor; NFκB, nuclear factor κB; JNK, Jun N-terminal kinase; JBD, JNK-binding domain; TBS, triethanolamine-buffered saline; TBSST, TBS containing 0.1% Tween 20; EGF, epidermal growth factor; CBP, CREB binding protein; KID, kinase-inducible domain; PC, prostate carcinoma; LUC, luciferase; wt, wild type; CAM, calcein AM.

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3-phosphoinositide-dependent kinase 1/Akt activation has been implicated in regulating sensitivity to cytotoxins, such as tumor necrosis factor (TNF-α), which induce apoptosis (27–29). Although the transcription factor nuclear factor κB (NFκB) is partly responsible for cellular responses to TNF-α through NFκB protein interactions with GSK-3β (30), other transcription factors also may be affected by TNF-α, particularly those influenced by Akt suppression of GSK-3β activity.

We conducted the present study to 1) determine the effects of alleviating GSK-3β suppression by Akt and assess GSK-3β function in prostate cancer cells, 2) examine the phosphorylation of CREB on Ser139 by GSK-3β in intact cell nuclei and its effects on CRE-mediated transcription, and 3) examine the intracellular distribution of GSK-3β mutants that were altered at critical phosphorylation sites.

EXPERIMENTAL PROCEDURES

**Cells and Culture Conditions—**PC-3 human prostate adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). These cells were maintained in a 1:1 (vol:vol) mixture of Dulbecco’s minimal essential medium and F-12 medium supplemented with 10% fetal bovine serum.

**Reagents—**Recombinant TNF-α, IGF-1, and epidermal growth factor (EGF) were purchased from R&D Systems Inc. (Minneapolis, MN). Anti-Tyr(P)32/33-GSK-3β anti-β-catenin (Upstate Biotechnology, Inc., Lake Placid, NY), anti-GSK-3β-specific, anti-Ser(P)9-GSK-3, and anti-Ser(P)9-Jun (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were purchased from the respective manufacturers. Rabbit anti-Ser(P)9-CREB antibody was purchased from Upstate Biotechnology. Anti-Ser(P)9/GSK-3 and anti–Jun antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies were purchased from Pierce. Goat anti-mouse and goat anti-rabbit Alexa 488-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR).

**Preparation of Anti-CREIELS328RRPS333Y-CREB Antibody—**Rabbit polyclonal antibodies were raised against a diphosphorylated CREB peptide CRKREELSS328RRPS333Y. The diphosphopeptide CRKREELSS328RRPS333Y was conjugated with a keyhole limpet hemocyanin carrier by generating a sulfi de linkage using maleimidem unsaturated chemistry, and a diphosphopeptide antibody was purified on a CRKREELSS328RRPS333Y affinity column. A second affinity purification was carried out using a monophosphorylated peptide CRKREELSRPS333Y affinity column to eliminate antibodies that interacted with the monophosphorylated peptide. The resulting passage through fraction produced an anti-phosphopeptide antibody that was high titer (1:1,753,000) based on a peptide linked-enzyme immunoassortant assay that failed to bind CRKREELSRPS333Y but could specifically recognize both CREIELLS328RRPSYR and CRKREELSS328RRPS333Y.

**GSK-3β Assays—**PC-3 cells were incubated overnight in serum-free Dulbecco’s minimal essential medium/F-12 medium and then incubated with or without 10 ng/ml TNF-α for 20 min. The cells were harvested, and GSK-3β was immunoprecipitated from detergent lysates using an anti-GSK-3β antibody (Transduction Laboratories, Los Angeles, CA). GSK-3β kinase assays were then carried out as described by Pap and Cooper (17) using a Ser(P)9-CREB peptide (amino acids 125–135; New England Biolabs, Beverly, MA) in the presence of γ-[32P]ATP (specific activity, 3000 Ci/mm; Amersham Biosciences). Cells were treated with 20 μM LY294002 (Sigma), a PI3K-specific inhibitor, to inhibit Akt and isolate GSK-3β in an active form. The results were normalized as a relative cpm incorporated into a CREB peptide, compared with untreated control samples, and analyzed for statistical significance using the StatView software program (SAS Institute, Inc., Cary, NC).

**Nuclear Extracts—**Nuclear extracts were prepared as described by Schaefer et al. (31). Briefly, after cells were washed with cold phosphate-buffered saline wash solution (phosphate-buffered saline containing 0.1% BSA complete protease mixture tablet (Roche Molecular Biochemicals) and 10 μg Na3VO4) of the following conditions: 1 mg of hypotonic buffer (10 mM Hepes, pH 8.0, 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol, 25 mM NaF, 1 mM Na3VO4, and 1% BSA complete protease mixture inhibitors) and incubated on wet ice for 10 min. The resuspended lysate was centrifuged at 7500 rpm at 4°C for 5 min. The nuclear suspension as washed 2× and centrifuged at 12,000 rpm for 5 min. Nuclear preparations were examined by light microscopy for the presence of cytosolic components, which were not present. The nuclear pellets were lysed with sodium dodecyl sulfate sample buffer and DNA sheared through a 20-gauge needle. These lysates were then compared with total cell lysates for the presence of actin. The actin traces of actin were present in the nuclear lysate preparations, whereas the actin content was very high in the total cell lysate (data not shown).

**Western Blot Analysis—**PC-3 cells were left untreated or treated with 10 ng/ml TNF-α, 1 ng/ml IGF-1, 10 ng/ml EGF, or 1 μM insulin for 16 h at 37°C and subsequently analyzed for expression and activation of phospho–GSK-3β. Whole-cell or nuclear lysates were made from cell samples according to a previously described method (31). Proteins were separated on a NuPAGE 6–12% gradient gel (Novex, Carlsbad, CA), electrotransferred to a nitrocellulose membrane, and immobilobloted with a primary antibody overnight at 4°C. Immune complexes were visualized using SuperSignal chemiluminescence (Pierce).

**Transfection and Luciferase (LUC) Assays—**The commercially available plasmids used were pCRE-LUC, pCIScCK (an inactive LUC control) (Stratagene, La Jolla, CA), and pAP1-LUC, (BD Biosciences Clontech, Palo Alto, CA). The pCMV-4 control plasmid was a gift from D. Russell. The plasmids were generated in the authors laboratories and included pCDNA3-Flag-JBD, pCNG/GSK-3wt, pCGN/GSK-3ΔI, and pCGN/GSK-3ΔII (29). Cells were plated at a density of 400,000 cells per well, using six-well plates. The next day, FuGENE 6 (Roche Diagnostics, Indianapolis, IN) was used to transfect cells in accordance with the manufacturer’s instructions. Each plasmid concentration was 1 μg/ml, unless stated otherwise.

To normalize LUC activity either pCRE-LUC or pAP1-LUC was transfected in excess of 6-fold (0.86 μg/ml) greater than Renilla-LUC (0.13 μg/ml; pTK-LUC, Promega, Madison, WI). The transfected cells were harvested and analyzed using the Firelight system (a gift from Corin Rich and Packard Instrument Co., Naperville, IL) in accordance with the manufacturer’s instructions. Quantification was performed in a 96-well black plate using a TOPCOUNT multwell plate scintillation counter (Packard Instrument Co., in the photon-counting mode). All pCRE-LUC or pAP1-LUC DNA were normalized to Renilla-LUC that was co-transfected to determine the effect on the relative promoter activity. To establish if there was any change in the level of pCRE-LUC and pAP1-LUC DNA in PC-3 cells or those cotransfected with GSK-3β LUC, DNA template levels were determined by PCR using primers designed to amplify LUC. The levels of LUC DNA were the same in the cells transfected with either pCRE-LUC or pAP1-LUC DNA alone or when LUC reporter constructs were cotransfected with any of the GSK-3β expression vectors (data not shown).

**Densitometry—**These experiments were performed multiple times and quantified by densitometry, and these data were subjected to statistical analysis. All densitometric analyses were performed using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and the corresponding software program, ImageQuant (Molecular Dynamics). Images were quantified using NIH Image 1.62 (National Institutes of Health), and statistical analysis was performed using StatView 5.01 (SAS Institute Inc.). The density of the proteins analyzed was normalized to the appropriate controls (e.g., actin). Student’s t test was used to determine the significant differences between the mean relative densities of the various experimental groups, represented as p values.

**Immunofluorescence—**Immunofluorescence analysis was performed as described previously (35). Briefly, cells were grown on laminin-coated coverslips, which were used for TNF-α treatment. The coverslips were then fixed with 1% paraformaldehyde and permeabilized using 1% Nonidet P-40 detergent. Next, samples were blocked with 3% bovine serum albumin solution in triethanolamine-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T). A primary rabbit polyclonal antibody recognizing GSK-3β, Ser(P)9-CREB or Ser(P)9-Ser(P)323-CREB was diluted (1:200 vol/vol) in TBS-T containing 1% bovine serum albumin. Coverslips were then rinsed with TBS and blocked with the secondary antibody Alexa 488 (Molecular Probes) in TBS-T. After the cells were washed with secondary antibodies, counterstaining was performed using 500 nm 4’,6-diamidino-2-phenylindole dilactate (Molecular Probes) to identify the nuclei and 1 unit/ml Alexa 594-phaloidin (Molecular Probes) in TBS-T at 4°C to stain the cytoskeleton. The samples were then rinsed and mounted on glass slides using Prolong antifade solution (Molecular Probes). The slides were then analyzed via epifluorescence microscopy, and data were acquired using digital image analysis as described previously (35).
Cell Growth—Cell growth was measured using the vital dye calcein AM (CAM ester, Molecular Probes), which is membrane-permeable and nonfluorescent before activation by nonspecific esterases within viable cells. The cleavage product CAM emits green fluorescence. Cells transfected with either GSK-3β expression vectors or control vector were plated in 96-well plates and incubated with CAM ester in HEPES-buffered saline solution for 15 min at 25 °C. Fluorescence was quantified using a BioRad 9600 plate reader at 488 nm, and data were analyzed using the Statview software program (SAS Institute, Inc.).

Immunostaining of Paraffin Sections for Ser(D)216-Ser(D)217-CREB—

Immunohistochemistry was carried out in paraffin-embedded sections (5 μm) after deparaffinization and rehydration in xylene, graded alcohol, and phosphate-buffered saline, respectively. The endogenous peroxidase activity was quenched by incubating the sections in 0.3% hydrogen peroxide for 20 min at room temperature. After blocking the sections with 1.5% normal horse serum in phosphate-buffered saline for 1 h, anti-Ser(D)216-Ser(D)217-CREB was applied to the sections and incubated overnight at 4 °C. Then the sections were incubated with the appropriate biotinylated secondary antibody and ABC-AP reagent according to the manufacturer's instructions (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA). Peroxidase activity was detected with an AP dye reagent (3,3'-diaminobenzidine tetrahydrochloride containing 0.02% of hydrogen peroxide for 10 min. The sections were counterstained with hematoxylin and mounted with coverslips.

Molecular Modeling—Structural data obtained from the Protein Data Bank code 1KDX (3β) were used to generate a model of CREB-kinase inducible domain (KID) and CBP-KID interaction domain interactions. Biotechnix 3d software (Gentech, Parc Sophia Antipolis, France) was used for three-dimensional rendering of CREB on a G4 Titanium Powerbook Computer (Apple Computer Corp., Cupertino, CA).

RESULTS

GSK-3β Activity Is Suppressed in PC-3 Cells—Endogenous GSK-3β enzymatic activity was low in PC-3 cells (Fig. 1A, lane 1) as determined by the immune complex kinase assay using CREB peptide as the substrate. This low GSK-3β activity in untreated cells correlated with a relatively high level of phosphorylation on Ser9 (Fig. 1B, lane 1). Because PI3K is involved in the activation of Akt, which in turn phosphorylates GSK-3β at Ser9 and, thus, inactivates it, we treated cells with LY294002, a PI3K inhibitor, to determine whether GSK-3β is suppressed through this pathway. LY294002 increased GSK-3β activity—2-fold (Fig. 1A, lane 2), and this effect was enhanced by combining LY294002 with TNF-α (Fig. 1A, lane 4). This increase in enzyme activity correlated with a decrease in the phosphorylation of Ser9 with LY294002 alone (Fig. 1B, lane 2), which decreased further when LY294002 treatment was combined with TNF-α (Fig. 1B, lane 8). Furthermore, PC-3 cells were treated with increasing concentrations of LY294002, and then enzymatic activity was determined by CREB peptide phosphorylation along with measuring the phosphorylation of Ser9 by Western analysis. After densitometric quantification, regression analysis was performed using the Statview program to compare the relative GSK-3β enzymatic activity in relation to the inverse value of the mean phospho-Ser9 density. An R² value approximately equal to 0.9 indicated that the relative activity of GSK-3β decreased in conjuction with increasing the phosphorylation of GSK-3β on Ser9. In addition, two PI3K stimulators, EGF and IGF-1 (Fig. 1B, lanes 3 and 4, respectively), did not further suppress GSK-3β activity by increasing the phosphorylation of GSK-3β at Ser9 beyond control levels. Similar studies showed that these treatments caused a decrease in β-catenin levels (Fig. 2). Furthermore, results similar to those observed for EGF and IGF were obtained using insulin (data not shown). These results indicate that endogenous GSK-3β activity is largely suppressed by the PI3K pathway in proliferating PC-3 cells and that activation of GSK-3β enhanced the breakdown of β-catenin.

GSK-3β Stimulated CRE-mediated Transcription and Suppressed Activator Protein-1 (AP1)-mediated Transcription—CREB and Jun family members can take part in binding to and activating CRE-containing promoters; Jun family members can also activate AP1 transcription response elements. To determine how CRE and AP1 transcriptional activities were affected by GSK-3β, PC-3 cells were cotransfected with GSK-3β, with either the CRE or AP1 response element-containing luciferase reporter constructs (CRE-LUC and AP1-LUC, respectively).

The CRE-LUC activity in PC-3 cells cotransfected with GSK-3β was 6-fold higher than that in cells transfected with CRE-LUC alone (Fig. 3). The LUC vector without the CRE elements (pCISCK) showed no LUC activity, and the expression vector pCMV4 without the GSK-3 CDNA did not affect LUC activity (data not shown). We also observed that in the absence of cotransfected GSK-3β, AP1-LUC activity was about 20 times higher than for CRE-LUC. In contrast to its effect on CRE-LUC activity, GSK-3β strongly inhibited AP1-LUC activity. These data indicate that overexpression of GSK-3β can differentially regulate CREB-mediated and AP1-mediated activity in PC-3 cells.

Phosphorylation of GSK-3β Variants—We next investigated the overexpression of GSK-3β proteins that contained various point mutations or deletions to determine how these molecular changes affected the protein phosphorylation patterns and the intracellular distribution of GSK-3β (Fig. 4). Deletions of the first nine amino acids of GSK-3β results in the GSK-3β9 protein, which is constitutively active and cannot be inactivated by PI3K/Akt. Ser9 phosphorylation was present on all forms of GSK-3β except on the GSK-3β9 transfectants (Fig. 4A, Δ9 lane lower band is absent). In contrast, GSK-3βY216F contains a mutation of tyrosine Tyr216 to a phenylalanine, resulting in a dominant-negative-acting protein that cannot activate CREB. Tyr216 was phosphorylated on endogenous GSK-3β in PC-3 cells (Fig. 4A, lane C) and on the wild-type (wt) and Δ9-transfected forms of GSK-3β. The total Tyr216 phosphorylation was reduced in the GSK-3βY216F transfectants, which included some Tyr216 phosphorylation on endogenous GSK-3β, since the phenylalanine on GSK-3βY216F cannot be phosphorylated (Fig. 4A, lane Y216F).

Intracellular Distribution of GSK-3β Variants—Examination of the intracellular distribution of the different GSK-3β proteins by immunofluorescence using a GSK-3β-specific antibody showed that untransfected and empty vector-transfected control cells contained low levels of endogenous GSK-3β in both the cytoplasm and nucleus of PC-3 cells (Fig. 4B, control (C)). Exogenous, wt GSK-3β (when overexpressed) was found in both the cytoplasm and the nucleus of PC-3 cells (Fig. 4B, wt). The GSK-3βY216F protein was predominantly found in the cytoplasm, and the GSK-3β9 protein was almost exclusively nuclear (Fig. 4B, Y216F and Δ9).

β-Catenin Expression Decreased in PC-3 Cells Transfected with Active Forms of GSK-3β—In PC-3 cells that were transfected with inactive GSK-3β (Y216F) there was no change in β-catenin protein levels, whereas transfection of enzymatically active GSK-3β wt and Δ9 constructs caused a decrease in β-catenin protein levels (Fig. 5).

Active GSK-3β Expression Suppresses Cell Growth—Forced expression of wt GSK-3β suppressed growth by 12% and the GSK-3β9 by 33%, whereas the expression of empty vector and GSK-3βY216F had no effect (Fig. 6). These data indicate that the overexpression of enzymatically active GSK-3β proteins suppresses the prostate cell growth.

GSK-3β-stimulated CRE-LUC Activity—We further examined GSK-3β function in PC-3 cells by assessing the effects of transfected wt GSK-3β, GSK-3β9, and GSK-3βY216F constructs on the CRE-LUC reporter activity (Fig. 7A). Transfected wt GSK-3β slightly increased CRE-mediated activity in PC-3 cells. The dominant-negative-acting GSK-3βY216F did not
stimulate CRE, whereas constitutively active GSK-3β/H9252 caused a dramatic 80-fold increase in CRE activity.

Effects of Totally Suppressing Jun Transactivation on CRE-LUC Activity—Jun can increase CRE promoter activity but to a lesser extent than CREB (37). Because CREB and Jun can both activate CRE, we examined GSK-3β/H9252 effects on CRE transactivation in the absence of Jun activity. To achieve this condition we attempted to eliminate Jun transactivation in PC-3 cells. The phosphorylation of Jun at Ser63 by Jun N-terminal kinase (JNK) controls the transactivation state of Jun (38, 39). By limiting Jun phosphorylation at Ser63, it should be possible to limit the effects of Jun on CRE. The JNK binding domain (JBD) fragment of the scaffold protein Jun-interacting protein-1 competitively binds JNK and prevents its activation in cells (32, 40). JBD was expressed in PC-3 cells to sequester JNK and, thereby, minimize its ability to transactivate Jun (Fig. 7B). When JBD was coexpressed with wt GSK-3β/H9252 in PC-3 cells, it doubled the level of CRE-LUC activity. GSK-3β/Y216F activity of Ser(P)9 using a Student's t test. Statistical analysis showed a significant difference between control samples and TNF-α-treated samples (a, p < 0.004) and between control samples and all of the LY294002-treated samples (b, p < 0.001).

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alone or when combined with JBD did not stimulate CRE. However, the combination of GSK-3β9 and JBD hyperactivated CRE-LUC activity to the highest level that we observed (130-fold). These data suggest that Jun, which is a weaker activator of CRE-driven transcription than CREB itself, when fully suppressed (by combining GSK-3α-activated CRE-LUC activity to the highest level that we observed (130-fold). These data suggest that Jun, which is a weaker activator of CRE-driven transcription than CREB itself, when fully suppressed (by combining GSK-3β-activating CREB plus JBD). The level of Ser(P)9 alone was significantly increased when compared with control but the level was completely eliminated by JBD. These phosphorylation levels were amplified further after TNF-α treatment. In contrast to the effects on CREB phosphorylation patterns in PC-3 cells, phosphorylation of Jun on Ser63 was not strongly suppressed by transfection with GSK-3β9 alone but was completely eliminated by JBD, consistent with the idea that expression of JBD inactivates Jun (data not shown).

Effects of GSK-3β9 Alone or in Combination with JBD on Intracellular Distribution of Ser(P)129-CREB and Ser(P)133-CREB—We used our affinity-purified antibodies to localize and determine the phosphorylation status of Ser(P)129-CREB and Ser(P)133-CREB in cells transfected with GSK-3β9 alone or combined with JBD (Fig. 8B). The level of Ser(P)133-CREB in the nuclei of PC-3 cells did not change significantly under any conditions examined. In contrast to Ser(P)133-CREB, Ser(P)129-CREB labeling was absent from control PC-3 cell samples, but nuclear labeling of Ser(P)129-CREB increased when GSK-3β9 alone was transfected into PC-3 cells or combined with JBD. These data indicate that overexpression of the constitutively active GSK-3β9 protein in PC-3 cells leads to phosphorylation of Ser129 on CREB in the cell nuclei after CREB has already been prephosphorylated at Ser133. (P)S129-(P)S133-CREB Is Present in the Nuclei of Prostate Tissue Samples—Paraffin-embedded prostate tissue samples were analyzed by immunohistochemical staining for Ser(P)133-CREB.
cells were transfected with various GSK-3\(\beta\) mutants. PC-3 cells were transfected with control, empty vector (lane 1), or various GSK-3\(\beta\) constructs (wt, \(\Delta9\), and Y216F (lanes 2–4), subjected to detergent lysis, and examined by Western analysis for \(\beta\)-catenin and actin. Two independent experiments were quantified by densitometry, and the density of \(\beta\)-catenin was normalized to actin. Statistical analysis was performed using Student’s t test for the mean relative density of \(\beta\)-catenin. Statistical analysis showed a significant difference between control samples and wt-transfected samples (a, \(p < 0.006\)) or control samples and cells transfected with the \(\Delta9\) deletion construct (b, \(p < 0.006\)).

**Discussion**

GSK-3\(\beta\) is a primary target of Akt, which inhibits GSK-3\(\beta\) function by phosphorylating it on Ser\(^9\) in proliferating cells (16–19). Although many studies indicate that Akt is highly active in cancer (15), it is mainly by inference that GSK-3\(\beta\) activity has been thought to oppose Akt function in cancer cells. Our approach was to elevate GSK-3\(\beta\) activity in prostate cancer cells and examine the consequences of this activation. Our data show that basal levels of GSK-3\(\beta\) kinase activity were low in association with the high levels of GSK-3\(\beta\) phosphorylation on Ser\(^9\). We successfully increased the enzymatic activity of GSK-3\(\beta\) in PC-3 cells by decreasing the phosphorylation of GSK-3\(\beta\) on Ser\(^9\) through various methods, including the inhibition of PI3K by LY294002 treatment (Fig. 1, A and B) or TNF-\(\alpha\) treatment (Fig. 1, A and B) or the forced expression of a Ser\(^9\) deletion mutant form of GSK-3\(\beta\) that was constitutively active (Fig. 4A). Because GSK-3\(\beta\) phosphorylates and thereby regulates the functions of numerous proteins involved in signaling, metabolism, and protein synthesis and structural proteins (1, 3, 4), we examined certain transcription factors as downstream targets of activated GSK-3\(\beta\).

To determine the downstream consequence of increasing GSK-3\(\beta\) activity, we examined the phosphorylation of CREB and its mediation of CRE transcriptional activity in PC-3 cells. We chose to examine CREB because the phosphorylation of CREB on Ser\(^\>\) by GSK-3\(\beta\) and activation of CRE transcription have been demonstrated in vitro but not in intact cells (3). Our data show that the ability of GSK-3\(\beta\) to phosphorylate the CREB nuclear transcription factor in PC-3 cells depends not only on increasing GSK-3\(\beta\) activity but also on the presence of GSK-3\(\beta\) in the nucleus. We showed that CREB is fully activated by serial phosphorylation beginning at Ser\(^\>\) and then at Ser\(^\>\) by constitutively active GSK-3\(\beta\) in cell nuclei (Fig. 8B).
The greatest increases in CRE-mediated LUC reporter activity occurred when active forms of GSK-3β were present in PC-3 cell nuclei. GSK-3β<sup>Δ9</sup> was the strongest stimulator of CRE-driven LUC reporter activity (80-fold, Fig. 7A), and GSK-3β<sup>Δ9</sup> was found almost exclusively in the nucleus (Fig. 4B). In contrast, GSK-3β<sup>Y216F</sup> failed to activate the CRE-LUC reporter (Fig. 7A) and did not enter the nucleus (Fig. 4B). The presence of GSK-3β in the nucleus was influenced by phosphorylation on Tyr<sup>216</sup>, and the activation state of the enzyme was controlled by phosphorylation on Ser<sup>9</sup>. Phosphorylation on Tyr<sup>216</sup> and Ser<sup>9</sup>...
was important for determining whether GSK-3β could phosphorylate CREB in the cell nucleus.

Although we determined that constitutively activated GSK-3β was available in the nucleus to interact with preactivated Ser(P)₁²⁹-CREB, the critical question remains as to whether Ser₁²⁹ is available as an exposed substrate for GSK-3β. Based on structural analysis, it is likely that Ser₁²⁹ on CREB is available for GSK-3β phosphorylation during CREB interactions with CREB-binding protein in transcriptional complexes. Ser(P)₁²⁹-CREB occurs in the exposed peptide loop at the junction of the KID αA helix (Fig. 10, A and B) (36). This position is adjacent to the Ser(P)₁³³-CREB priming site, which is at the αB helix of the KID domain. The presence of GSK-3β in the nucleus and the availability of Ser₁²⁹-CREB as a substrate support a role for GSK-3β in catalyzing the phosphorylation of Ser₁²⁹-CREB in cells.

GSK-3β inhibits the activity of many nuclear proteins, including Jun. The prime exception is CREB, which we show is activated in intact cells by GSK-3β through the phosphorylation of Ser¹²⁹. In PC-3 cells, GSK-3β appears to control the balance between Jun and CREB-mediated transcriptional activity. Transfecting PC-3 cells with constitutively active GSK-3β plus JBD shifted the balance of CRE-LUC-mediated transcriptional activity from Jun- to CREB-dominated transcription. JBD has been shown to selectively inhibit JNK activity and prevent Jun and/or ATF-2 transactivation (32, 40).

When we cotransfected PC-3 cells with JBD and GSK-3β, basal-level CRE activity increased 130-fold (Fig. 7B). In this experiment, Jun was expected to simultaneously lose both DNA binding activity (because of phosphorylation by GSK-3β) and transcriptional transactivating activity (because of JNK suppression by JBD). Our observation of suppressed Jun phosphorylation in the presence of JBD was consistent with this expectation and indicated that suppressed JNK activity plus elevated GSK-3β activity minimizes Jun activity in PC-3 cells.

We have additional support for the involvement of CREB phosphorylation by GSK-3β in prostate cancer from the examination of prostate cancer tissue samples. These data show that the phosphorylation of Ser(P)¹²⁹-Ser(P)¹³³-CREB is high in low grade (well-differentiated) prostate cancer but decreases in high grade (poorly differentiated) cancer, corresponding to a decrease in GSK-3β activity. These findings suggest that Ser(P)¹²⁹-Ser(P)¹³³-CREB may be associated with differentiation status in these tissues. Furthermore, CREB-driven elevation of CRE transcriptional activity is known to enhance cell differentiation, which often involves the suppression of cell growth (41–43). The growth of PC-3 cells was significantly suppressed when enzymatically active GSK-3β was overexpressed in these cells (Fig. 6). Little is known about the genetic markers that are associated with prostate cell differentiation, but the usefulness of differentiating agents in prostate cancer therapy is well-recognized (44). Although CREB activation may induce PC-3 cells to differentiate, further investigation will be necessary to determine the gene expression patterns that are involved in this process.

GSK-3β inhibits the stability of many proteins, including β-catenin levels, through the influence of the Wnt pathway in prostate cancer.

**Fig. 9. Ser(P)¹²⁹-Ser(P)¹³³-CREB in the nuclei of prostate tissue samples.** Paraffin-embedded prostate tissue was analyzed by immunohistochemical staining for Ser(P)¹²⁹-Ser(P)¹³³-CREB. Ser(P)¹²⁹, Ser(P)¹³³-CREB expression was high in the nuclei of low grade prostate cancer (well-differentiated) (A) but decreased in high grade (poorly differentiated) prostate cancer (B), corresponding to a decrease in GSK-3β activity that is expected to occur in advanced stage prostate cancer.

**Fig. 10. Molecular models of Ser¹²⁹-CREB availability.** A and B illustrate that Ser¹²⁹ is available for phosphorylation by GSK-3β at the junction of the αB helix and the exposed peptide loop of the CREB-KID domain. The Ser¹²⁹ structure at the αA helix junction of the CREB kinase-inducible domain (KID) domain is exposed for phosphorylation after priming by Ser(P)¹³³-CREB and binding to the CBP-KID interaction domain. Ser¹²⁹ and primed Ser(P)¹³³ are illustrated by van der Waals radii. A, ball-and-stick illustration emphasizes the position of Ser(P)¹³³ (phosphorous in yellow, oxygen in red). B, stick illustration emphasizing the position of Ser¹²⁹ and α-helix structures (pink). The structural data used to generate this model of CREB-KID and CBP-KID interaction domain interactions are available under Protein Data Bank code 1KDX (36).
cancer cells. GSK-3β phosphorylates β-catenin on Ser-33 and -37 and Thr-41. This primes β-catenin for ubiquitination and lysis by proteasomes (45, 46). Mutations of these Ser/Thr phosphorylation sites in β-catenin prevent proteolysis and cause the accumulation of β-catenin/TNF-α complex in nuclei, which activates transcription of a variety of genes (45, 46). These mutations in β-catenin are reported to occur in 5% of prostate cancers (47, 48). However, many prostate cell lines, including the PC-3 line, do not contain these mutations (47, 48). The overexpression of wt GSK-3β or the constitutively active GSK-3β mutant with PI3K inhibitor LY294002 all caused β-catenin levels to decrease (Figs. 3 and 5). These data indicate that β-catenin was normally regulated in PC-3 cells and not likely to affect transcriptional activity in these studies.

Our data also help establish a link between TNF-α responsiveness and PI3K/3-phosphoinositide-dependent kinase 1/Akt activation during the regulation of GSK-3β function. In studies by Hoeflich and coworkers (30), the loss of GSK-3β caused embryonic lethality in knockout mice and involved liver degeneration that correlated with hypersensitivity to TNF-α. TNF-α caused the transcriptional activation of NFκB through a GSK-3β-dependent mechanism, but the GSK-3β phosphorylation that correlated with hypersensitivity to TNF-α decreased (27–29). We show that treating PC-3 cells with TNF-α increases GSK-3β enzymatic activity, which synergizes with LY294002 treatment (Fig. 1A). Consistent with increases in GSK-3β enzymatic activity, TNF-α treatment alone or in combination with LY294002 treatment decreased the phosphorylation of GSK-3β on Ser9 (Fig. 1B). TNF-α also increased Ser(P)129-Ser(P)133-β-catenin on Ser-33 and -37, and Thr-41. This primes β-catenin for ubiquitination and lysis by proteasomes (45, 46). Mutations of these Ser/Thr phosphorylation sites in β-catenin prevent proteolysis and cause the accumulation of β-catenin/TNF-α complex in nuclei, which activates transcription of a variety of genes (45, 46). These mutations in β-catenin are reported to occur in 5% of prostate cancers (47, 48). However, many prostate cell lines, including the PC-3 line, do not contain these mutations (47, 48). The overexpression of wt GSK-3β or the constitutively active GSK-3β mutant with PI3K inhibitor LY294002 all caused β-catenin levels to decrease (Figs. 3 and 5). These data indicate that β-catenin was normally regulated in PC-3 cells and not likely to affect transcriptional activity in these studies.

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