Glycogen Synthase Kinase-3 Represses Cyclic AMP Response Element-binding Protein (CREB)-targeted Immediate Early Genes in Quiescent Cells

John W. Tullai1, Jie Chen1, Michael E. Schaffer1, Eliza Kamnetzky1, Simon Kasif3, and Geoffrey M. Cooper1,2

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From the 1Department of Biology, the 2Bioinformatics Program, and the 3Department of Biomedical Engineering, Boston University, Boston, Massachusetts 02215

Despite its central role in cell survival and proliferation, the transcriptional program controlled by GSK-3 is poorly understood. We have employed a systems level approach to characterize gene regulation downstream of PI 3-kinase/Akt/GSK-3 signaling in response to growth factor stimulation of quiescent cells. Of 31 immediate-early genes whose induction was dependent on PI 3-kinase signaling, 12 were induced directly by inhibition of GSK-3. Most of the GSK-3-regulated genes encoded transcription factors, growth factors, and signaling molecules. Binding sites for CREB were highly over-represented in the upstream regions of these genes, with 9 genes containing CREB sites that were conserved in mouse orthologs. Binding sites predicted in 6 genes were confirmed by CREB chromatin immunoprecipitation and forskolin induction of CBP binding. Moreover, CREB siRNA substantially blocked induction of 5 genes by forskolin and of 3 genes following inhibition of GSK-3. These results indicate that GSK-3 actively represses gene expression in quiescent cells, with inhibition of CREB playing a key role in this transcriptional response.

The protein-serine/threonine kinase glycogen synthase kinase-3 (GSK-3)3 plays a central role in a wide variety of normal and pathological cellular processes. First described as the kinase capable of phosphorylating and inactivating glycogen synthase, GSK-3 is now recognized as a key regulator of many normal processes such as cellular development, proliferation, and survival. Dysregulation of GSK-3 activity has been linked to various human diseases including Alzheimer’s disease, diabetes, cancer, heart disease, schizophrenia, and mood disorders.

GSK-3 can be regulated either by Wnt signaling or by the phosphatidylinositol (PI) 3-kinase/Akt pathways. Wnt signaling leads to an inhibition of the phosphorylation of β-catenin by GSK-3 and a corresponding increase in transcriptional activation of β-catenin/Tcf target genes (1). Activation of PI 3-kinase in response to stimulation of cells by insulin or growth factor causes a decrease in GSK-3 activity because of an inhibitory phosphorylation by Akt of GSK-3β on serine 9 or GSK-3α on serine 21 (2). The resulting inhibition of GSK-3 plays a critical role in signaling cell growth and survival downstream of PI 3-kinase. In the absence of growth factor stimulation, GSK-3 is active and can inhibit cell proliferation as well as induce apoptosis (3–6). The substrates of GSK-3 that have been implicated in regulation of cell proliferation and survival include the translation initiation factor eIF2B (7, 8), cyclin D1 (3), the Bcl-2 family member Mcl-1 (9), and a variety of transcription factors that are phosphorylated by GSK-3 either in vitro or in vivo (5, 10). However, the role of GSK-3 in control of gene expression downstream of PI 3-kinase/Akt signaling is not well understood.

We have combined global gene expression profiling with computational analysis to characterize gene regulation downstream of PI 3-kinase/Akt/GSK-3 signaling. We previously identified immediate early genes that were induced by the PI 3-kinase/Akt signaling pathway in response to growth factor stimulation of quiescent cells (11). In the present study, we have characterized a subset of these genes that are regulated by GSK-3, and by combining computational predictions with experimental analyses we have identified CREB (cyclic AMP response element-binding protein) as a key transcription factor responsible for gene regulation by GSK-3.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments**—T98G human glioblastoma cells were grown in Minimal Essential Medium (Invitrogen) containing 10% fetal calf serum. For inhibitor and growth factor treatments, cells were incubated in serum-free medium for 72 h, and left untreated or treated with 50 ng/ml human PDGF-BB (Sigma) for 30 min, 10 μM forskolin (Sigma), or Me2SO vehicle for 1 h, or 5 μM SB-216763 (BioMol) or Me2SO vehicle for 1 h, as indicated in the text.

**GSK-3 Kinase Assays**—GSK-3 kinase activity was assayed using a crude cell lysate method (12) with modifications. Briefly, T98G cells were rinsed with ice-cold phosphate-buffered saline and lysed using a Dounce homogenizer in 8 mM...
MOPS, pH 7.4, 0.2 mM EDTA, 10 mM magnesium acetate, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5 mM benzamidine. Samples were then centrifuged at 10,000 × g for 10 min at 4 °C. 12.5-μl aliquots of the supernatant (containing ~150 ng of total protein) were then incubated at 30 °C for 30 min in 50 μl of reaction buffer (5 mM MOPS, pH 7.4, 0.1 mM EDTA, 20 mM magnesium chloride, 250 μM [γ-32P]ATP (1.6 Ci/mol), and 62.5 μM of either a GSK-3 peptide substrate (YRRAAVPSSPLSRHSSPHQ(pS)EDEEE) or a control pseudosubstrate peptide (YRRAAVPSSPLSRHSSPHQALEEDE) (Upstate Biotechnology). Reactions were stopped by spotting the sample onto P81 phosphocellulose paper for 30 s, followed by washing each filter for 5 min in 40 ml of 0.75% phosphoric acid (three times), and once with acetone. Filters were allowed to dry and incorporated 32P measured by scintillation counting. Incorporation into control pseudosubstrate samples was subtracted as background.

Quantitative RT-PCR—Total RNA preparations were used in quantitative real-time reverse transcription polymerase chain reactions (RT-PCR). Reverse transcription of 0.25 μg of total RNA was performed in 20 μl using SYBR green RT-PCR reagents and random hexamer primers (Applied Biosystems) as recommended by the manufacturer. Following a 95 °C incubation for 10 min, forty cycles of PCR (95 °C/15 s; 60 °C/1 min), were then performed on an ABI Prism 7900HT Sequence Detection System with 1 μl of the RT reaction, 100 nM PCR primers (see supplemental Table S1 for primer sequences), and SYBR Green PCR Master Mix in 5-μl reactions. Threshold cycles (Ct) for three replicate reactions were determined using Sequence Detection System software (version 2.2.2) and relative transcript abundance calculated following normalization with a GAPDH PCR amplicon. Amplification of only a single species was verified by a dissociation curve for each reaction.

Identification of Transcription Factor Binding Sites and Statistical Analysis of the Site Frequencies—To identify candidate regulators of the SB-216763-induced genes, the web-based statistical analysis of the site frequencies (match) was verified by a dissociation curve for each reaction. 

The sum of false negative and false positive error rates (min-SUM) were considered matches. For each matrix, the distribution of sites per gene in the SB-216763-induced gene set was compared with the distribution detected in a previously described background set of genes that were expressed in T98G cells, but not induced by PDGF (11). The significance of site frequencies relative to background was assessed with a one-sided permutation test using the exactRankTests package in R (16), and p values were adjusted with a false discovery rate (FDR) correction (17). Only 165 matrices for the human-only set and 162 matrices for the human-mouse conserved set (those meeting the criteria of less than 5 hits per 5 kb of upstream sequence in the background set and at least one hit in 50% of the test genes) were considered in the correction calculations.

Identification of Conserved Binding Site Enrichment—5-kb upstream human sequences (version hg17, May 2004) and orthologous mouse genomic sequences (version mm5, May 2004) were extracted from 8-way 5-kb upstream MULTIZ alignments obtained from the University of California Santa Cruz Genome Browser Data Base. The human and mouse sequences were independently scanned with Match as described above and predicted sites in human and mouse at the same alignment position were considered matches. Statistical analysis and p value corrections were performed as described above.

TATA Box Identification—To identify transcripts with an upstream TATA box, sequences −55 to −5 relative to the transcription start site were scanned with the Match algorithm (14) using a TBP binding site matrix (supplemental Fig. S2A). Subsequences with at least one site scoring greater than a threshold were considered TATA sites and those with at least one site in the corresponding aligned mouse sequence were considered conserved. Using a set of sequences bound by human TATA-binding protein (hTBP) in vitro (18), the threshold was based on the collection of highest scoring positions within each sequence. These 68 sequences, derived from a restriction endonuclease protection selection and amplification (REPSA) assay, were grouped into four categories based on their consensus sequence as TATAAA, TAAATA, TATATA, or Other. A threshold of ≥0.7 was selected to identify at least 95% of the RESPA sequences in the TATAAA, TATATA, and TAAATA groups (supplemental Fig. S2B). This threshold identified TATA sites upstream of 20.68% of 16,743 unique human RefSeq transcripts (hg17) (supplemental Fig. S2C).

Chromatin Immunoprecipitation—Chromatin immunoprecipitations were performed as described (11), except that cells were resuspended in lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, and aprotinin) for 10 min after formaldehyde fixation. Crude nuclei were collected by microcentrifugation at 4000 × g for 3 min, washed once with ice-cold 1× phosphate-buffered saline, and resuspended in high salt lysis buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, and aprotinin). After sonication to obtain an average of 500-bp chromatin fragments, samples were pre-cleared with sonicated salmon sperm DNA/protein A agarose (50% slurry), and immunoprecipitated overnight at 4 °C using

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6.25 μg/ml anti CREB-1 antibody (Santa Cruz Biotechnology, sc-186), 3.3 μg/ml anti CBP antibody (Santa Cruz Biotechnology, sc-369) or equivalent concentrations of normal rabbit IgG (Santa Cruz Biotechnology sc-2027) as negative control. For CREB ChIP, beads were washed three times in low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl, pH 8.1), once in LiCl wash (0.25 M LiCl, 1% IGEPLAL-Ca 630, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and twice with 1× TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). For CBP ChIP, beads were washed successively in low salt wash, high salt wash (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl, pH 8.1), LiCl wash, and twice in 1× TE. Immunoprecipitated chromatin was quantified with real-time PCR (see Fig. 4 for mapped amplicons and supplemental Table S1 for primer sequences). GAPDH was used as a negative control for CREB ChIPs (19).

RNA Interference—RNA interference transfections were conducted using HiPerfect reagent (Qiagen) and pre-designed siRNAs (final concentration 10 nM, Ambion). Transfection mixtures were prepared in 400 μl of total volume by adding 24 μl HiPerfect and either CREB1 siRNA (Ambion siRNA ID 109994, 5′-GGUGGA AAAUGGACUGGC UTT-3′) or nonspecific siRNA (Ambion negative control siRNA 1, catalog 4611) to serum-free medium, mixed, and incubated at room temperature for 10 min. The transfection mixtures were then overlaid with 4.6 ml of 105 cells/ml in serum-containing medium and transferred to 60-mm plates. Cells were incubated at 37°C, 5% CO2 for 48 h and then serum-starved for 25 h prior to treatment with forskolin or SB-2167623 for 1 h. Cells were then harvested, total RNA-extracted, and analyzed by real time RT-PCR.

**RESULTS**

Identification of Genes Induced by GSK-3 Inhibition—Microarray analysis was previously used to identify immediate-early genes that were induced by stimulation of quiescent T98G human glioblastoma cells with PDGF (11). Inhibitors of PI 3-kinase (LY294002) and MEK (U0126) were then used to identify genes whose induction in response to PDGF stimulation was dependent on either PI 3-kinase or MEK/ERK signaling. This analysis revealed four categories of genes: genes that were induced independent of PI 3-kinase and MEK/ERK, genes that were dependent on PI 3-kinase, genes that were dependent on MEK/ERK, and genes that were dependent on both PI 3-kinase and MEK/ERK signaling (Fig. 1) (11). We have investigated 31 of these genes (10 that were dependent only on PI 3-kinase and 21 that were dependent on both PI 3-kinase and MEK/ERK signaling) to determine whether they could be induced in serum-starved, quiescent T98G cells by treatment with 0.15 mM siRNA (GenBankTM), so that computational analysis of transcription factor binding sites could be conducted.

The effect of the GSK-3 inhibitor SB-216763, which inhibits both the α- and β-isoforms (20), was first tested by assaying lysates of T98G cells for GSK-3 activity (Fig. 2). Quiescent T98G cells were treated with SB-216763, vehicle (Me₂SO) or PDGF, and GSK-3 activity determined by *in vitro* kinase assays (12) (Fig. 2A). As expected, quiescent T98G cells exhibited the highest GSK-3 activity. When cells were treated for 1 h with SB-216763, activity decreased to 39% (±10%, n = 3) of the activity of quiescent cells treated with vehicle alone. Similarly, quiescent cells treated with PDGF demonstrated an activity decrease to 53% (±8%, n = 3) of the activity of quiescent, untreated cells. These results are consistent with previous studies on the effects of both growth factor stimulation and SB-216763 treatment (2, 6, 21) and indicate that treatment with SB-216763 inhibited GSK-3 activity comparably to PDGF. In contrast, treatment of quiescent cells with SB-216763 did not significantly affect either Akt or ERK activity (Fig. 2B).

Gene induction by GSK-3 inhibition (with SB-216763) was compared with induction by PDGF by quantitative real time RT-PCR (Fig. 3). Quiescent cells were treated with SB-216763 for 60 min in the absence of growth factor stimulation, as compared with cells stimulated with PDGF for 30 min. Overall, 12 of the 31 PI 3-kinase-regulated genes tested were induced greater than 2-fold upon direct GSK-3 inhibition in the absence of any growth factor stimulation, ranging from 2-fold (21) to nearly 16-fold (20). Regarding the distribution across the two groups of genes, 4 of 10 genes in the PI 3-kinase-only-dependent group were induced more than 2-fold by SB-216763 and 8 of 21 genes in the PI 3-kinase and MEK/ERK-dependent group were induced more than 2-fold by SB-216763. Typical of immediate-early genes, 11 of the GSK-3-regulated genes encoded transcription factors (*FOSB, NR4A1, NR4A2, NR4A3, BHLHB2*), growth factors (*CYR61, CCL8, CTGF*), and signaling molecules (*RGS1, RGS2, ARHE*). For some genes, such as *CYR61, RGS2, CCL8, BHLHB2, PLAU*, and *ARHE*, the induction by SB-216763 alone was comparable to their induction by PDGF. This result suggested that GSK-3 was a major contributor to the regulation of these genes.
Identification of Transcription Factor Binding Sites in SB-216763-induced Genes—Co-regulated genes often share common transcription factor binding sites. Therefore, the twelve genes up-regulated greater than 2-fold from Fig. 3 were examined for common cis-elements to identify transcription factors that might be targeted by GSK-3 signaling. Predicted cis-elements are generally more likely to be physiologically relevant if they are evolutionarily conserved in multiple organisms and can be demonstrated to occur in an aligned upstream sequence from an orthologous gene (22, 23). Therefore, each 5-kb upstream human sequence, as well as the corresponding orthologous mouse sequence, was analyzed with the Match program using 546 vertebrate matrices from TRANSFAC Professional (v8.4). All sites with a score above the recommended threshold to minimize the sum of false negative and false positive (minSUM) hits were recorded, and the frequency of predicted transcription factor binding sites was examined across the group of twelve SB-216763-induced genes and compared with a background set of genes that were expressed in T98G cells but were not induced by PDGF (11). The most significantly scoring matrices are shown in Table 1 (see supplemental Table S2 for complete analysis). When considering only the human sequences (Table 1A), three matrices were significantly overrepresented in the twelve genes at p < 0.05: V$CREB_Q3, V$CREB_Q2_01, and V$CREBATF_Q6, all representing CREB binding sites. The same three CREB matrices scored in the top four most highly significant sites that were conserved between human and mouse, in addition to a matrix representing binding sites for CEBPα (Table 1B).

We previously identified binding sites for CREB as being over-represented in both PI 3-kinase and PI 3-kinase/ERK-regulated genes (11). It was therefore of interest to compare the frequency of predicted CREB binding sites in the GSK-3-regulated genes to the 19 PI 3-kinase-regulated genes that were not induced by GSK-3 inhibition (see Fig. 3). For these 19 genes, neither matrices for CREB nor any other transcription factor were over-represented in the human sequences alone. When considering sites that were conserved between human and mouse, the V$CREB_Q3 matrix was among the top ten sites predicted in these 19 genes, but with a p value that was only marginally significant (5.57 × 10⁻³), compared with p = 1.47 × 10⁻⁶ in the GSK-3-regulated genes. Over-representation of CREB binding sites is thus specific for the subset of GSK-3-regulated genes.

The over-representation of CREB binding sites in GSK-3-regulated genes is consistent with CREB being a known target of GSK-3 (5). Therefore, we further explored the possible involvement of CREB in the induction of these genes. The alignments of the 5-kb upstream regions, and the distribution of predicted CREB binding sites are compiled in Fig. 4 (see supplemental Fig. S1 for other predicted binding sites). Consistent with its role as a putative regulator for this cluster, CREB binding sites were predicted in 11 of 12 genes. Despite low overall percent identities, conserved CREB sites between mouse and human are predicted in 9 of 12 genes within the 5-kb window.

To further corroborate the prediction of CREB as a major regulator of this group of genes, we separately searched for TATA motifs, as others have shown a proximal TATA box is often present in the promoter region of CREB-activated genes (19). Interestingly, 6 of 9 SB-216763-induced genes with conserved CREB sites (67%) also contained a proximal TATA box.
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FIGURE 3. Gene induction by inhibition of GSK-3. T98G cells were serum-starved for 72 h rendering them quiescent. Cells were then treated with PDGF-BB (50 ng/ml) for 30 min, or SB-216763 (5 μM) for 1 h, and were compared with untreated and vehicle-treated cells (Me2SO), respectively. Total RNA was isolated and measured using quantitative real time RT-PCR. We analyzed induction of 31 of the 34 PI 3-kinase-de-activated genes indicated in Fig. 1, because there were 3 genes for which adequate quantitative real time RT-PCR primers could not be designed (see supplemental Table S1 for primer sequences). Data are the means of two experiments ± S.E.

TABLE 1
Summary of transcription factor matrices with over-represented binding sites in the upstream sequences of SB-216763 up-regulated genes

| Matrix             | p-value      |
|--------------------|--------------|
| V$CREB_Q3         | 1.08 × 10^{-2} |
| V$CREB_Q2_01      | 1.17 × 10^{-2} |
| VSREBATF_Q6       | 3.80 × 10^{-3} |

A. Human only

B. Human-Mouse conservation

| Matrix             | p-value      |
|--------------------|--------------|
| V$CREB_Q3         | 1.47 × 10^{-6} |
| V$CREB_Q2_01      | 2.04 × 10^{-5} |
| V$CREBPA_01       | 3.73 × 10^{-5} |
| V$CREBATF_Q6      | 1.12 × 10^{-4} |
| V$HNF3_Q6         | 1.87 × 10^{-4} |
| V$OCCT1_Q5_01     | 1.87 × 10^{-4} |
| V$PAI3            | 2.18 × 10^{-4} |
| V$SAP1_Q4_01      | 2.68 × 10^{-4} |
| V$SAP1_Q6_01      | 2.68 × 10^{-4} |

(Fig. 4), compared with an occurrence of only 20.68% for 16,743 genes encoding unique human RefSeq transcripts (supplemental Fig. S2).

Forskolin Induction of GSK-3 Target Genes—Genes that have functional CREB binding sites in their upstream regions are likely to be induced by stimulation of protein kinase A with cyclic AMP. To test this, quiescent T98G cells were subjected to forskolin treatment to stimulate cyclic AMP-mediated pathways, and the transcripts of the 12 GSK-3-regulated genes were measured by quantitative real time RT-PCR (Fig. 5). Forskolin up-regulated 9 of 12 genes greater than 2-fold, consistent with CREB-mediated regulation of these genes. The values ranged from just over 2-fold for BHLHB2 (2^1), to more than 380-fold (2^8.6) for NR4A2. In most (7 of 9) cases, forskolin induction was consistent with the presence of CREB sites that were conserved in mouse. BHLHB2, FOSB, NR4A1, NR4A2, NR4A3, RGS1, and RGS2 all exhibited greater than 2-fold inductions by forskolin, and likewise contained evolutionarily conserved binding sites within the 5-kb window (Fig. 4).

Binding of CREB and CBP to GSK-3 Target Genes—To gain a better understanding of the CREB regulation of the SB-216763 up-regulated genes, CREB binding to upstream regions containing the predicted sites in 10 GSK-3 target genes was tested by ChIP (Fig. 6A). These 10 genes included the 9 genes with predicted CREB binding sites that were conserved in the mouse, as well as CTGF, which was also forskolin-inducible. Serum-starved T98G cells were treated with SB-216763 or vehicle (Me2SO), followed by immunoprecipitation of chromatin using an anti-CREB1 antibody. Quantitative real time PCR was then used to detect specific upstream regions (amplicons for all ChIPs are mapped in Fig. 4). As expected, because CREB has been shown to occupy its binding sites constitutively (19), the vehicle-treated and SB-216763-treated samples showed similar levels of precipitation by the anti-CREB antibody (Fig. 6A). Comparable results were also obtained with untreated control cells (data not shown).

The upstream regions of 6 genes (FOSB, NR4A1, NR4A2, NR4A3, RGS2, and CYR61[II]) were specifically immunoprecipitated by anti-CREB antibody (0.1–0.6% of input) compared with control IgG, whereas a negative control promoter (GAPDH) was not immunoprecipitated (<0.02% of input). This is consistent with the finding that FOSB, NR4A1, NR4A2, NR4A3, and RGS2 were induced by forskolin (Fig. 5). One of two regions upstream of CYR61 (amplon II, Fig. 4) was also immunoprecipitated by anti-CREB antibody, despite the lack of induction of CYR61 by forskolin, consistent with the observation that not all genes with CREB occupancy are physiologically...
active (24). In contrast, CREB did not bind to upstream regions of RGS1, BHLHB2, PLAU, and CTGF, either at the sites shown in Fig. 6A or the additional sites shown in Fig. 4. These genes included one that was not induced by forskolin (PLAU) as well as three that were forskolin-inducible (RGS1, BHLHB2, and CTGF).
The activation of CREB-inducible genes in response to cAMP is mediated by recruitment of the coactivator CBP (25, 26), which binds to CREB phosphorylated at serine 133 (the target of protein kinase A). We therefore performed ChIPs with an anti-CBP antibody to examine CBP recruitment to the upstream regions of the six genes to which CREB binding had been demonstrated. CBP ChIPs were performed on both control and forskolin or SB-216763-treated cells, and the data are presented as the fold increase of CBP binding to each promoter following either forskolin or SB-216763 treatment (Fig. 6B). Forskolin treatment resulted in a significant increase in CBP binding (2–5-fold) to the predicted sites of all five of the forskolin-induced genes (FOSB, NR4A1, NR4A2, NR4A3, and RGS2) as well as to CYR61, even though CYR61 was not induced by forskolin. Treatment with SB-216763 similarly resulted in increased binding of CBP to the upstream regions of FOSB and NR4A1 (2- and 3-fold, respectively), indicating that GSK-3 inhibition also resulted in CBP recruitment to these promoters.

**Effect of CREB RNAi on the Induction of GSK-3-regulated Genes**—We next sought to determine whether CREB was required for the activation of these genes using RNAi, which knocked down CREB greater than 90% in T98G cells (Fig. 7A). The effect of CREB knockdown was determined for both forskolin and SB-216763 induction of the 9 genes that were induced by forskolin, as well as for SB-216763 induction of CYR61. Treatment with CREB siRNA decreased forskolin induction of FOSB, NR4A1, NR4A2, NR4A3, and RGS2 by 60% to more than 90%, indicating that CREB was required for forskolin induction of these genes (Fig. 7B). In contrast, CREB RNAi did not significantly affect forskolin induction of RGS1, CTGF, CCL8, or BHLHB2 (Fig. 7B). These results are consistent with CREB occupancy and CBP recruitment to the upstream regions of FOSB, NR4A1, NR4A2, NR4A3, and RGS2, but not RGS1, CTGF, CCL8, or BHLHB2 (see Fig. 6). Knockdown of CREB similarly resulted in an 80–100% decrease in the induction of NR4A1, NR4A3 and FOSB by SB-216763 (Fig. 7C), directly demonstrating that CREB is required for induction of these genes following inhibition of GSK-3.

**DISCUSSION**

GSK-3 can be regulated either by Wnt signaling or by the PI 3-kinase/Akt pathway. The role of GSK-3 in the Wnt pathway is well characterized, with GSK-3 acting to control gene expression by phosphorylating β-catenin (1). As a target of PI 3-kinase/Akt signaling, GSK-3 plays a key role in regulating cell metabolism, proliferation, and survival. However, although its targets include a variety of transcription factors, the transcriptional program controlled by GSK-3 downstream of PI 3-kinase/Akt signaling has not been elucidated. In the present study, we have employed a systems level approach to understanding the GSK-3 transcriptional program. We began with a set of 31 immediate-early genes we had previously characterized by global expression profiling as being induced by PI 3-kinase signaling downstream of PDGF stimulation of quiescent cells (11). GSK-3 is active in quiescent cells and is inhibited by Akt phosphorylation, thus we sought to identify GSK-3-regulated genes by determining whether any of the PDGF-inducible PI 3-kinase-dependent genes could be induced simply by inhibiting GSK-3 in the absence of growth factor stimulation. 12 of these 31 PI 3-kinase-dependent genes (~40%) were inducible by the direct inhibition of GSK-3 with the small molecule inhibitor SB-216763 in the absence of growth factor. These results indicate that the activity of GSK-3 plays a major role in sensing the repression of genes in quiescent cells.

Using computational methods, we then examined the upstream sequences of the 12 genes induced by GSK-3 inhibition. Our analysis focused on prediction of transcription factor binding sites that were over-represented in the group of GSK-3-regulated genes and were conserved between mouse and human orthologs. This analysis predicted binding sites for CREB as the most significantly overrepresented and evolutionarily conserved sites among the upstream sequences of GSK-3-regulated genes. This prediction is consistent with previous studies showing that CREB is phosphorylated by GSK-3 (27). Moreover, the activity of CREB is generally inhibited by GSK-3 (28–31), consistent with the induction of CREB-regulated genes following GSK-3 inhibition.

The upstream regions of 11 of 12 GSK-3-regulated genes contained predicted CREB binding sites, and 9 of the 12 contained predicted CREB sites that were conserved between mouse and human. CREB binding to the upstream regions of 6 of these 9 genes was demonstrated by ChIP, providing experimental
confirmation of the computational predictions. These results confirm previous demonstrations of CREB binding sites upstream of NR4A1, RGS2, and FOSB (24, 32), as well as identifying new CREB binding sites upstream of CYR61, NR4A2, and NR4A3.

Proximal TATA boxes have been reported to be frequently present in the promoter regions of CREB-regulated genes (19). Overall, 6 of 9 SB-216763 induced genes with conserved CREB sites contained TATA boxes in their promoter regions, as compared with only about 21% of human transcripts in RefSeq (see supplemental Fig. S2C). Notably, conserved TATA boxes were found upstream of 5 of 6 genes to which CREB binding was demonstrated by ChIP. The combined prediction of both a conserved CREB site and a TATA box was thus highly correlated with CREB binding to the upstream region of a gene.

The physiological activity of these CREB binding sites was demonstrated by treatment of cells with forskolin, which activates the cAMP pathway. Forskolin induced transcription of 5 of the 6 genes with CREB binding sites (FOSB, NR4A1, NR4A2, NR4A3, and RGS1). In addition, forskolin led to recruitment of the CREB coactivator CBP to all 5 of these genes, as well as to the 6th gene to which CREB binding had been demonstrated (CYR61). It is interesting that CREB bound to the CYR61 upstream region, and that CBP binding was increased in response to forskolin, even though CYR61 was not induced by forskolin. Although CYR61 appears to contain a functional CREB site, it thus appears that activation of CREB is not sufficient for CYR61 induction.

Inhibition of GSK-3 also induced recruitment of CBP to the upstream regions of FOSB and NR4A1. GSK-3 phosphorylates CREB at serine 129, and increased binding of CBP to CREB has been previously observed in cells following inhibition of GSK-3 (31). It is also possible that phosphorylation by GSK3 affects the association of CREB with other coactivators such as p300 or members of the TORC family (33).

A direct role for CREB in induction of all 5 of the forskolin-inducible genes was further demonstrated by RNA interference experiments, in which knockdown of CREB substantially inhibited (60–90%) the induction of these genes by forskolin. Importantly, RNAi against CREB also significantly inhibited (>80%) the induction of 3 of these genes (NR4A1, NR4A3, and FOSB) resulting from inhibition of GSK-3. It is likely that CREB also contributes to induction of the other three genes following inhibition by GSK-3, but that knockdown of CREB is not sufficient to block induction because of the action of additional transcription factors targeted by GSK-3. Such factors might include members of the AP1 and CEBP families. Binding sites for these factors were also over-represented among the
GSK-3 inducible genes (see Table 1) and members of the AP1 and CEBP families have also been reported to be phosphorylated by GSK-3 (10, 34–36).

Taken together, these results indicate that CREB is a major target of PI 3-kinase/Akt/GSK-3 signaling during gene induction in response to growth factor stimulation. Previous studies have shown that regulation of CREB by GSK-3 plays a significant role in the Toll-like receptor inflammatory response.

FIGURE 7. Effect of CREB RNAi on gene induction. A, knockdown of CREB by RNAi. Cells were transfected with either CREB1 siRNA or nonspecific control siRNA and analyzed by immunoblots for CREB and β-actin. B, effect of CREB RNAi on forskolin induction. T98G cells were transfected with CREB1 or nonspecific control siRNA, serum-starved for 25 h, and then treated with either vehicle or forskolin for 1 h. Expression of the indicated genes was determined by real-time RT-PCR. Data are presented as average fold induction by forskolin (FK) as compared with vehicle-treated controls in cells that were transfected with CREB siRNA (gray bars) or nonspecific siRNA (black bars). Data are the mean of duplicate experiments ± S.E. C, effect of CREB RNAi on SB-216763 induction. Cells were transfected and analyzed as in B, except they were stimulated by treatment with SB-216763 for 1 h rather than forskolin. Data are the means of 2–4 experiments ± S.E.
downstream of PI 3-kinase in monocytes and macrophages (31). Our results extend the role of GSK-3/CREB signaling to the more general paradigm of gene regulation downstream of growth factor receptors. GSK-3 is active under conditions of growth factor deprivation, which induce cell cycle arrest and/or apoptosis. In quiescent cells deprived of growth factors, it appears that GSK-3 actively represses gene expression, with inhibition of CREB playing a key role in this transcriptional response.

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