THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 41, pp. 30479–30484, October 13, 2006
Printed in the U.S.A.

Differential Roles of Glycogen Synthase Kinase-3 Isoforms in the Regulation of Transcriptional Activation*

Received for publication, August 7, 2006 Published, JBC Papers in Press, August 15, 2006, DOI 10.1074/jbc.M607468200

Min-Huei Liang and De-Maw Chuang[1]

From the Molecular Neurobiology Section, National Institute of Mental Health, Bethesda, Maryland 20892-1363

Glycogen synthase kinase-3 (GSK-3) exists as two structurally similar isoforms, α and β, whose activities are negatively regulated by serine phosphorylation but positively controlled by tyrosine phosphorylation. We used GSK-3 isoform-specific small interfering RNAs, dominant negative mutants, and pharmacological inhibitors to search for the differential roles for both GSK-3 isoforms in regulating transcriptional activation in cultured rat cerebral cortical neurons. GSK-3α and GSK-3β were shown to have differentially regulated transactivation such that GSK-3α silencing/inhibition was more robust than GSK-3β silencing/inhibition in causing cAMP-responsive element- and NF-κB-dependent transactivation. Moreover, protein-DNA array studies identified two novel GSK-3-regulated transcription factors, early growth response 1 and Smad3/4, which were oppositely affected by GSK-3α or GSK-3β silencing or inhibition. Taken together, our results underscore critical variations in the function and regulation of GSK-3α and GSK-3β. The development of GSK-3 isoform-specific inhibitors is thus crucial for therapeutic intervention of GSK-3-related neuropathological conditions.

Gkycogen synthase kinase 3 (GSK-3)[2] has a central role in regulating cellular function, structure, and survival by coordinated control of phosphorylation of GSK-3 itself and its substrates, the formation of substrate-specific GSK-3 binding/scaffolding protein complexes, and GSK-3 subcellular distribution (reviewed in Refs. 1, 2). Among the substrates phosphorylated by GSK-3 are a wide spectrum of transcription factors, including CREB, NF-κB, AP-1, heat shock factor 1, β-catenin, T-cell factor (TCF)/lymphoid enhancer factor, p53, and Bax (reviewed in Ref. 3). Dysregulation of GSK-3-mediated signaling pathways and substrate phosphorylation has been implicated in the pathophysiology of mood disorders (4, 5), schizophrenia (6), Alzheimer disease, type II diabetes, and cancer, among others (2).

GSK-3 is an evolutionary conserved, constitutively active, serine/threonine kinase that consists of two distinct isoforms, α and β, in mammals (7). Their activities are regulated by serine and tyrosine phosphorylation (8, 9). GSK-3α and GSK-3β are highly homologous within their kinase domains and display similar biochemical and substrate properties (2). However, some structural differences between the two isoforms exist in their N- and C-terminal regions, and a number of reports suggest that the regulation and functions of both GSK-3 isoforms are not always identical. For example, GSK-3α, but not GSK-3β, is regulated by protein kinase C isozymes (10), whereas GSK-3β is more potent than GSK-3α in phosphorylating phosphatase inhibitor 2 (11). The disruption of the GSK-3β isoform in mice results in embryonic lethality, and the GSK-3α isoform is unable to compensate for the loss of GSK-3β (12). Additionally, transfection and small interfering RNA (siRNA) studies suggest that GSK-3α is involved in the processing of β-amyloid precursor protein to form $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides, whereas the role of GSK-3β is unclear (13, 14). Moreover, differential transcriptional and translational regulation for these two isoforms might be due to variable expression of GSK-3α and GSK-3β in different mammalian tissues (7). Transcriptional activation regulates the expression of many downstream genes of signaling pathways directly by GSK-3 through phosphorylation or indirectly as a consequence of GSK-3 activity changes. Although differential regulations of transcription factors by the two isoforms of GSK-3 were proposed (11- 13), the distinct mechanisms and endogenous targets of such regulations remain to be investigated.

In the present study, we employed transfection of GSK-3 isoform-specific siRNA, wild-type GSK-3α and -3β, and their dominant negative mutants to address their differential regulation of transactivation in cultured rat cerebral cortical neurons. The efficacies of both GSK-3 isoforms in regulating various transcription factors were compared, and new GSK-3-targeted transcription factors oppositely regulated by these two isoforms were identified.

MATERIALS AND METHODS

Rat Primary Cerebral Cortical Culture and Cell Culture for Human SH-SY5Y Neuroblastoma Cells—Cerebral cortical neurons prepared from 18-day-old embryonic rats were cultured as described previously (15) with modifications. Briefly, dissociated cortical cells were collected by centrifugation at 80 × *g* and seeded at a density of 1×10^6 cells/cm^2 on poly-D-lysine (Sigma)-coated culture plates. Cortical neurons were used for

* This work was supported by the Intramural Research Program at the National Institute of Mental Health (project number Z01MH002468-18). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: National Institute of Mental Health, Bldg. 10/Rm. 4C-206, 10 Center Dr., MSC 1363, Bethesda, MD 20892-1363. Tel.: 301-496-4915; Fax: 301-480-9290; E-mail: chuang@mail.nih.gov.

[2] The abbreviations used are: GSK, glycogen synthase kinase; AP-1, activator protein 1; CRE, cAMP-responsive element; CREB, CRE-binding protein; EGR-1, early growth response-1; GRE, glucocorticoid-responsive element; NF-κB, nuclear factor of κB; siRNA, small interfering RNA; TCF, T-cell factor; DIV, days *in vitro*.

siRNA transfection after 9 days *in vitro* (DIV) unless otherwise indicated. Human neuroblastoma SH-SY5Y cells were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% L-glutamine. Cells with a passage number ranging from 102 to 105 were used in the studies.

Plasmid DNA—Reporter plasmids pEgr1-Luc and pSmad3/4-Luc were obtained from Panomics (Redwood city, CA). Reporter construct pRL-TK was purchased from Promega (Madison, WI). TCF reporter plasmid and mutant Tcf reporter plasmid (TOPflash and FOPflash, respectively) were purchased from Upstate (Charlottesville, VA). Mercury Pathway Profiling Luciferase System 1 (Clontech, Palo Alto, CA) including reporter constructs pAP-1-Luc, pCRE-Luc, pGRE-Luc, pNF-κB-Luc, and pHSE-Luc was used in conjunction with the silencing of GSK-3 isoform in studies of transcriptional regulation. Wild-type GSK-3α and its dominant negative mutant (hGSK3α/pMT2 and hGSK3α/pMT2-KR, respectively) were obtained from Dr. Peter S. Klein (University of Pennsylvania) with the kind permission of Dr. James R. Woodgett (Ontario Cancer Institute, Toronto, Canada). Wild-type GSK-3β (pAdTrack-CMV-GSK-3β) was supplied by Dr. Ratan V. Bhat (AstraZeneca R&D, Södertälje, Sweden). Two dominant negative mutants (pAdTrack-CMV-GSK-3β-K85R and pAdTrack-CMV-GSK-3β-R96A) for GSK-3β were generated using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The GSK-3β wild-type and two dominant negative mutants were subsequently subcloned into pEGFP-C1 vector (Clontech) to obtain the three plasmids used in this study, pEGFP-GSK-3β, pEGFP-GSK-3β-K85R, and pEGFP-GSK-3β-R96A.

siRNA Preparation and GSK-3 Inhibitors—Several siRNAs targeting either isoform of GSK-3 were designed according to GenBank™ accession numbers (NM_017344 for rat GSK-3α and NM_032080 for rat GSK-3β; NM_019884 for human GSK-3α and NM_002093 for human GSK-3β) and chemically synthesized (Dharmacon, Chicago, IL). The target sequences for the rat-specific GSK-3 siRNAs are as follows: αP747, TCACTTTACCAAGGCCAAG; αP1269, CTTCAGTCCTGGTGAACTT; αP1375, GCTTTAACTGAGACTCAGA; βP555, CCTCTTGCTGGATCCTGAT; βP514, GGTCTACCTTAACCTGGTG; βP1093, GAACTGTCAAGTAACCCAC. The siRNA mixture specific to either GSK-3 isoform consists of the three species of siRNAs described above and was used in all silencing experiments. An siRNA control (CONTROL non-targeting siRNA 1; Dharmacon) was used as an RNA interference control for all siRNA transfection experiments in both rat neurons and human neuroblastoma cells. Two structurally similar ATP-competitive GSK-3 inhibitors, SB216763 and SB41286, were purchased from Tocris (Ellisville, MO).

Transfection Assays and Dual Luciferase Reporter Assay—Rat primary cortical cultures were evenly plated and cultured for 9 DIV before transfection for 48 h with 80 nM siRNA using 8 μl of siPORT Amine transfection reagent (Ambion) in one ml of the transfection mixture. Transfection of luciferase reporter constructs and co-transfections of reporter plasmids with GSK-3 expression vectors (wild-type or dominant negative mutants) into primary cortical neurons were established using an Amaxa Nucleofector (Amaxa, Cologne, Germany) in conjunction with a rat neuron Nucleofector kit (Amaxa) at the time of cell plating. Co-transfections of luciferase reporter plasmids and GSK-3 expression vectors or siRNAs into SH-SY5Y cells were carried out using Lipofectamine 2000 (Invitrogen). Dual luciferase assay was performed using the dual luciferase reporter assay system (Promega). Routinely, dual reporter-transfected cortical neurons were lysed in passive lysis buffer (Promega) and subjected to the detection of firefly luciferase activity and, subsequently, *Renilla* luciferase activity, which serves as an internal control. Luciferase activities were determined by using a plate-reading luminometer (Packard Bioscience Company, Downers Grove, IL), and the activities of the experimental reporter were normalized to the activities of the background *Renilla* luciferase.

Transcription Factor-DNA Array I Analysis—Cortical neurons at 9 DIV were transfected with siRNA for GSK-3α or GSK-3β for 48 h. Cells were collected and nuclear extracts were prepared using a nuclear extraction kit (Panomics) for analysis with the TranSignal™ protein-DNA array I (Panomics). The procedures were performed according to the manufacturer's protocol with slight modification in the horseradish peroxidase detection process in which DNA Thunder™ Chemiluminescence Reagent Plus (PerkinElmer) was used. Quantitative array results showing more than 2-fold alterations in transcription factor-DNA binding were selected as positive for further confirmation using the reporter activity assay.

Immunoblotting Analysis—Cortical neurons were washed twice with 5 ml of phosphate-buffered saline and harvested by scraping into cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with a protease inhibitor mixture (Roche Diagnostics). Protein concentrations were determined by BCA protein assay reagent kit (Pierce). Antibodies against PAI-1, β-actin, β-catenin, and Bcl-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against GSK-3α was obtained from Upstate. Antibodies against GSK-3α, pCREB(Ser133), and CREB were purchased from Cell Signaling Technology (Beverly, MA). ECL Plus Western blotting detection system (Amersham Biosciences) was used to obtain Western blot signals, and autoradiographs were scanned. Intensity of immunoblot signals was analyzed using Scion Image software (Scion Image for Windows, NIH).

Statistical Analysis—All results presented are mean ± S.E. from three to four independent experiments, with three or four replicates for each data point. The results were analyzed for statistical significance in GraphPad InStat (GraphPad, San Diego, CA) by Student's *t* test or one-way analysis of variance. In analysis of variance, a Q-Q plot was adopted for normal distribution tests.

RESULTS

We investigated whether the GSK-3 isoforms are differentially involved in the regulation of transcription factors known to be modulated by GSK-3. Isoform-specific rat GSK-3 siRNAs were designed and synthesized. Western blotting was performed to assess the silencing effects of siRNA for GSK-3α or GSK-3β. Transfection of cortical neurons with different amounts of siRNA cocktails for either GSK-3α or GSK-3β

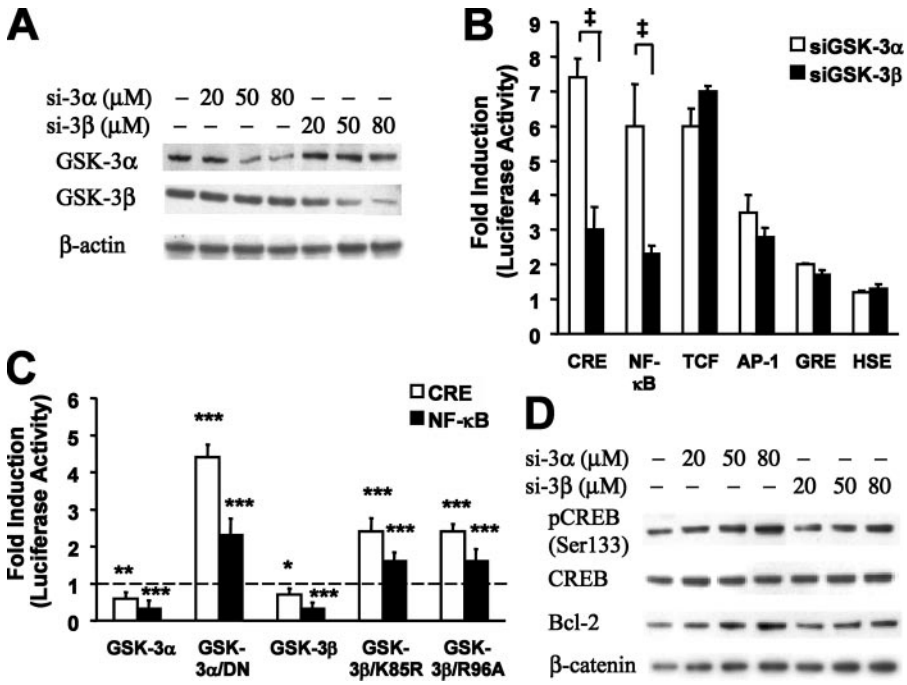

FIGURE 1. **Differential transcriptional activation of CRE and NF-κB signal pathways by GSK-3α or GSK-3β gene silencing.** *A,* representative Western blotting demonstration of selective reduction of GSK-3α and GSK-3β protein levels in cortical neurons transfected with indicated concentrations of GSK-3 isoform-specific siRNA cocktails for 48 h, starting from 9 DIV. β-actin was used as the loading control. *Si-3α* and *si-3β* represent treatment with isoform-specific siRNAs for GSK-3α and GSK-3β, respectively. Experiments were performed three times from independent cultures. *B,* differential transcription activation of CRE and NF-κB signal pathways in cortical neurons treated with 80 nM specific siRNA cocktails for GSK-3α or GSK-3β. siRNA cocktails specific to each GSK-3 isoform were used to transfect cortical neurons at 9 DIV, and the luciferase reporter assay was performed 48 h later. Luciferase activities are shown as means ± S.E. of -fold induction compared with the control from three independent experiments. ‡, $p < 0.05$, compared between siRNA for GSK-3α- and GSK-3β-treated groups *C,* GSK-3α and GSK-3β dominant negative mutants mimicked the differential effects of GSK-3 isoform silencing on transcriptional activation of CRE and NF-κB. Cortical neurons were transfected by Amaxa Nucleofector with either wild-type GSK-3α/GSK-3β or their dominant negative mutants, along with pNF-κB-Luc or pCRE-Luc reporter constructs at the time of cell plating. Luciferase activities are shown as means ± S.E. of –fold induction compared with the control from three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the non-targeting control. *D,* representative Western blotting demonstration of protein levels of pCREB(Ser133), CREB, Bcl-2, and β-catenin after treatment with GSK-3 isoform-specific siRNA cocktails for 48 h, starting from 9 DIV. Experiments were performed as described in *panel A.*

resulted in knockdown of their respective proteins 48 h after transfection, whereas protein levels of the non-targeted GSK-3 isoforms were unaffected (Fig. 1*A*). Treatment of cortical neurons with 80 nM siRNA cocktails for GSK-3α and GSK-3β caused 76 ± 14% ($n = 5$) and 81 ± 11% ($n = 5$) protein depletion of 3α and 3β isoforms, respectively. Thus, the magnitudes of protein reduction by specific siRNA treatments are similar for these two GSK-3 isoforms.

As measured by luciferase reporter assay, silencing GSK-3α and GSK-3β with their specific siRNAs was found to cause 7.5- and 3-fold activation of CRE-responsive transcription, respectively (Fig. 1*B*). NF-κB-responsive transcription was also more robustly activated by GSK-3α silencing than by GSK-3β silencing (6-fold *versus* 2-fold). Transcription of other responsive cis-element-containing reporter constructs, including TCF, AP-1, GRE, and HSE, was activated to various extents by GSK-3 silencing. Their degrees of activation were similar between the two isoforms using siRNA for either GSK-3α or GSK-3β.

By using dominant negative mutants of GSK-3α and GSK-3β, the differential activation of CRE- and NF-κB-dependent transcription by isoform-specific GSK-3 gene silencing was confirmed (Fig. 1*C*). Thus, a GSK-3α dominant negative mutant produced a greater transcriptional activation of CREB and NF-κB than two GSK-3β dominant negative mutants (GSK-3β K85R and GSK-3β R96A) (Fig. 1*C*). Conversely, transfection with either wild-type GSK-3α or wild-type GSK-3β resulted in the reduction of both CRE- and NF-κB-dependent transcriptional activities.

Transfection of cortical neurons with isoform-specific siRNAs for 48 h caused a modest increase in protein levels of pCREB(Ser133) and Bcl-2, a target gene of CRE-dependent transcription, in a concentration-dependent manner (Fig. 1*D*). Moreover, the increase of protein levels of both pCREB-(Ser133) and Bcl-2 was slightly greater in cortical neurons transfected with 80 nM GSK-3α siRNA compared with transfection with GSK-3β siRNA. These results are thus compatible with our observations indicating a higher magnitude of CRE-dependent transactivation resulting from GSK-3α gene silencing. On the other hand, a similar magnitude of a dose-dependent increase in the levels of β-catenin, a transcription factor associated with TCF or lymphoid enhancer factor 1 to regulate TCF-dependent transactivation, was observed in cortical neurons transfected with siRNA for either GSK-3 isoform (Fig. 1*D*). These results further support our observations that showed similar effects of protein depletion of either GSK-3 isoform on TCF-dependent transactivation (Fig. 1*B*).

Inhibition of both GSK-3 isoforms by two ATP-competitive GSK-3 inhibitors, SB216763 and SB415286, at various concentrations was carried out to examine the up-regulating effects of inhibitors on CRE- and NF-κB-dependent transactivation. As shown in Fig. 2*A*, we observed increased CRE-dependent transcription with a similar magnitude in a concentration-dependent manner. Comparable results were obtained for NF-κB-dependent transactivation (Fig. 2*B*). Treatment with GSK-3 inhibitors (3 μM SB216763 and 10 μM SB415286) resulted in modest activation of TCF-, AP-1-, GRE-, and HSE-responsive transcription in cortical neurons, supporting a regulatory role for GSK-3 in these four signaling pathways (Fig. 2*C*). Treatment with these two GKS-3 inhibitors resulted in an increase in β-catenin protein levels, suggesting that inhibition of GSK-3 kinase activity did occur (Fig. 2*D*). Transfection of cortical neurons with dominant negative mutants of either GSK-3 isoform also enhanced GRE-responsive transcription (data not shown),

confirming the inhibitory effect of GSK-3 in GRE-responsive signaling regulation.

Next, in an effort to search for novel targets of GSK-3, cortical neurons were transfected with siRNA specific for GSK-3α or GSK-3β, and the nuclear extracts were analyzed for protein-DNA binding activities. Changes of DNA binding activity with transcription factors EGR-1 (also known as nerve growth factor, NGF-1A) and Smad3/4 were noted (Fig. 3). Binding activity of EGR-1-responsive element with EGR-1 was down-regulated by transfection with siRNA for GSK-3α but up-regulated by siRNA for GSK-3β compared with the non-targeting siRNA control. Luciferase reporter assays confirmed that the transcriptional activity of EGR-1 was decreased by GSK-3α siRNA and its dominant negative mutant but increased by GSK-3β siRNA and its dominant negative mutants (Fig. 4*A*). In contrast, the DNA binding activity of Smad3/4 was up-regulated by GSK-3α siRNA and appeared to be unaffected by GSK-3β siRNA. Moreover, the activity of Smad3/4-responsive transcription was enhanced by GSK-3α siRNA and its dominant negative mutant but slightly reduced by GSK-3β siRNA and its R96A mutant (Fig. 4*B*). Of interest, treatment with the GSK-3 inhibitors SB216763 and SB415286 resulted in an increase in Smad3/4 transcriptional activities, similar to the effects of GSK-3α. Differential transcriptional regulation of EGR-1 and Smad3/4 by GSK-3 isoforms was also confirmed using isoform-specific siRNA and dominant negative mutants in human neuroblastoma SH-SY5Y cells (Fig. 4*C*) and further suggests that the opposite effects of these two isoforms in regulating transcription factors are not unique to cortical neurons.

The protein levels of plasminogen activator inhibitor type-1 (*PAI-1*), a Smad3/4-dependent TGF-β-regulated gene (16), was also differentially affected in cultured cortical neurons by GSK-3 isoform-specific siRNA gene silencing, similar to the effects on Smad3/4 signaling (Fig. 4*D*). Thus, *PAI-1* protein levels were up-regulated by GSK-3α siRNA but down-regulated by GSK-3β siRNA (Fig. 4*D*), even though the effect of transactivation by GSK-3β protein depletion was small in cortical cells (Fig. 4*B*). In addition, the transcription factor serum-responsive element was detected to be differentially up-regulated by GSK-3α siRNA and GSK-3β siRNA (Fig. 3). Experiments are in progress to verify this effect by reporter analysis.

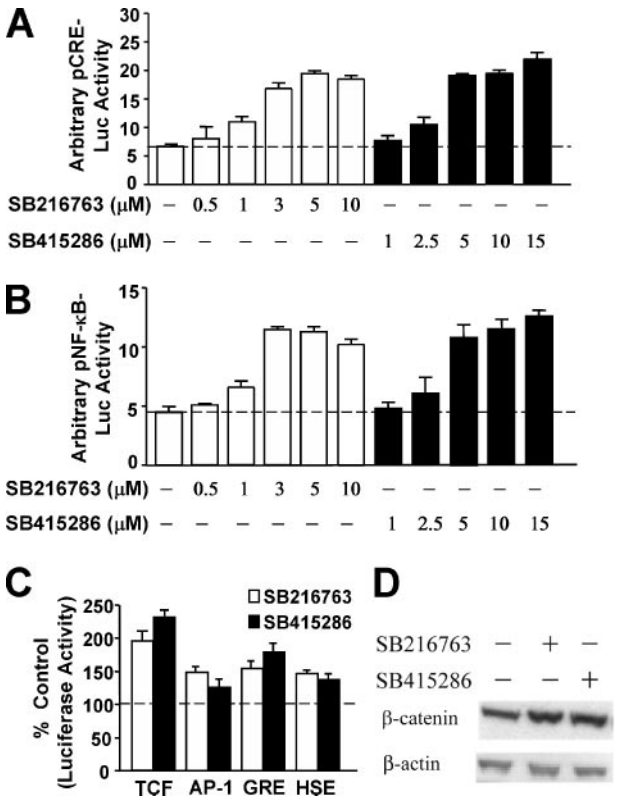

FIGURE 2. **Transcriptional activation of multiple signal pathways by the inhibition of GSK-3 using ATP-competitive inhibitors.** *A* and *B,* induction of transcription activation of CRE and NF-κB signal pathways in cortical neurons treated with specific GSK-3 inhibitors SB216763 and SB415286 at various concentrations for 72 h, starting from 8 DIV. Cell toxicity was not detected in the above doses tested by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay. Luciferase activities are shown as means ± S.E. of three independent experiments. *C,* induction of transcription activation of AP-1, GRE, and HSE signal pathways in cortical neurons treated with specific GSK-3 inhibitors SB216763 (3 μM) and SB415286 (10 μM) for 72 h, starting from 8 DIV. Luciferase activities are shown as means ± S.E. of percentage-induction compared with the control from three independent experiments. *D,* representative Western blots of β-catenin protein levels in cortical neurons treated with GSK-3 inhibitor SB216763 (3 μM) or SB415286 (10 μM) for 48 h starting from 9 DIV. All cultures were harvested at 11 DIV for Western blotting analysis. β-actin was used as the loading control. Experiments were performed three times using independent cultured cortical neurons.

DISCUSSION

The present study showed that inhibition of GSK-3α was more effective than that of GSK-3β in stimulating CREB- and

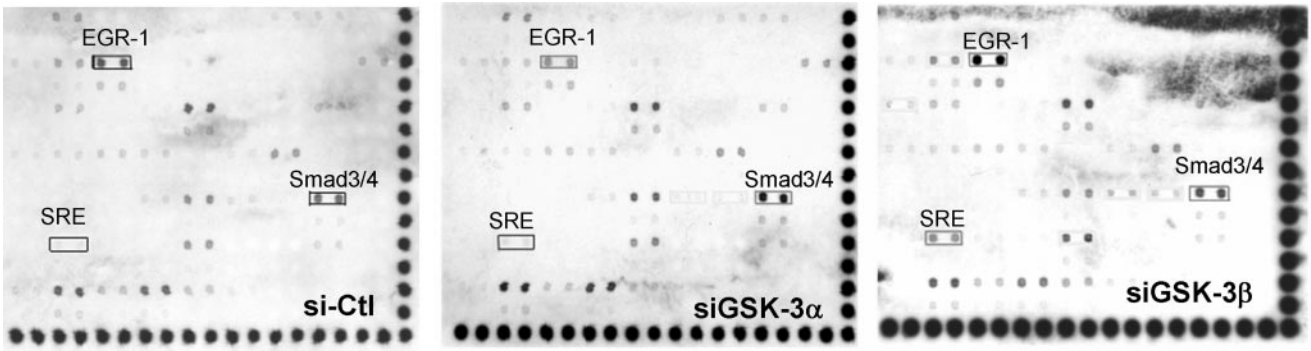

FIGURE 3. **Novel transcription factors differentially regulated by GSK-3 isoforms: A transcription factor-DNA array study.** TranSignal-DNA array indicated differential changes in transcriptional activity. Nuclear extracts from cortical neurons treated with 80 nM rat GSK-3 isoform-specific siRNAs at 9 DIV for 48 h were incubated with TranSignal biotin-labeled probe mix. After chemiluminescence detection, protein/DNA binding activities were identified. The *pairs of dots* marked in *boxes* indicate changes compared with the non-targeting control (*si-Ctl*).

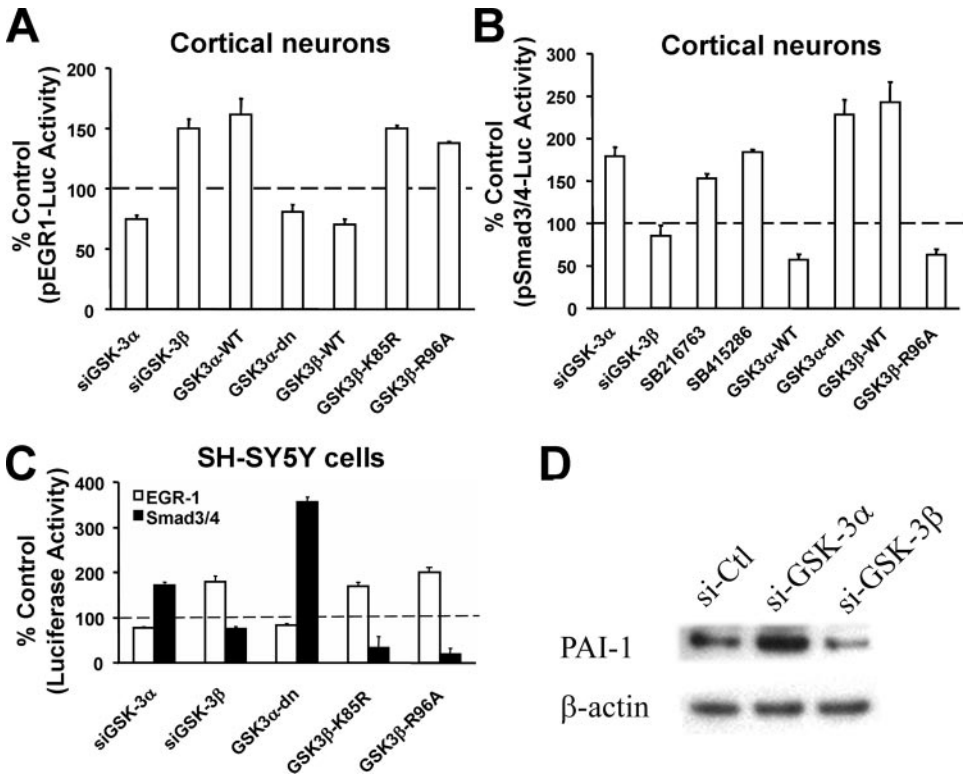

FIGURE 4. **Confirmation of differential regulation of EGR-1 and Smad3/4 transcriptional activities.** *A,* confirmation of EGR-1 transcriptional activation with luciferase reporter assay. Cortical neurons at the time of plating were co-transfected with either 4 μg of pEGR-1-Luc reporter construct and GSK-3 expression vectors (wild-type or mutant) or with 4 μg of a pEGR-1-Luc reporter construct followed by transfection with 80 nM siRNA mixture for GSK-3α or GSK-3β at 9 DIV. The luciferase activities of cell lysates were determined at 11 DIV. *B,* confirmation of Smad3/4 transcriptional activation with luciferase reporter assay. Cortical neurons were either co-transfected with 4 μg of pSmad3/4-Luc reporter construct and GSK-3 expression vectors and then treated with GSK-3 inhibitors (3 μM SB216763 or 10 μM SB415286) at 6 DIV for 5 days or transfected with 80 nM siRNA mixture for GSK-3α or GSK-3β at 9 DIV. The luciferase activities of cell lysates were determined at 11 DIV. *C,* confirmation of EGR-1 and Smad3/4 transcriptional regulation in SH-SY5Y human neuroblastoma cells. Cells were either co-transfected with reporter construct and GSK-3 expression vectors or co-transfected with reporter construct and human siRNA cocktails specific to either GSK-3 isoform at 9 DIV. The target sequences for those human-specific siRNA are: αP468, GTGGCTTACACGGACATCA; αP617, GCTGGACCACTGCAATATT; αP1132, CACCAACCCGGGAACAAAT; βP158, GTCAGCTATACAGACACTA; βP385, TCTGGTGCTGGACTATGTT; βP975, CTGCCCGACTAACACCACT. Luciferase activities of cell lysates were determined 48 h after transfection. Values shown represent means ± S.E. of % of control from three independent experiments. *D,* representative Western blotting analysis of protein levels of PAI-1 expression 48 h after siRNA transfection in cultured cortical neurons. β-actin was used as a protein-loading control. The *immunoblot* shown is representative of three experiments.

There are conflicting results regarding the functional effects of GSK-3 on the phosphorylation of NF-κB, with some showing activation (12, 23) and others reporting inhibition (24). It also should be noted that NF-κB activation is not always associated with survival proteins. A number of studies have demonstrated that NF-κB activation is linked to neuronal death induced by insults such as quinolinic acid excitotoxicity in the rat striatum (25) or cerebral ischemia in rodents (26). In addition, it has been reported that NF-κB is the only target known to be differentially regulated by two GSK-3 isoforms in a transgenic mouse model (12). The diversity of the cellular functions of NF-κB and the complexity of the regulation of NF-κB transcriptional activity by GSK-3 underscore the need for further studies to clarify the roles for GSK-3 isoforms in NF-κB regulation.

Our results suggest transcription factors Smad3 and EGR-1 as prospective substrates for GSK-3. Detection of potential phosphorylation sites by GSK-3 on EGR-1 and Smad3 using NetPhosK 1.0 server (27) further suggests possible direct regulation of these two transcription factors by GSK-3 (data not shown). Nine potential GSK-3β consensus phosphorylation sites of EGR-1 were found to reside on two main domains that are highly rich in serine/threonine residues. These sites are conserved among human, rat, mouse, and frog and constitute substrate recognition sites for GSK-3β. With Smad3, we found one tyrosine and three serine phosphorylation sites conserved among human, rat, mouse, frog, and fruit fly. Whether these two transcription factors are direct GSK-3 substrates and how they are regulated differentially by GSK-3 are currently being explored in our laboratory.

NF-κB-dependent transcriptional activation. Moreover, we demonstrated differential changes in the transcriptional activation of two transcription factors, EGR-1 and Smad3/4, in response to either GSK-3α or GSK-3β silencing using simultaneous multiple transcription factor/DNA array technology. This is, to our knowledge, the first evidence demonstrating the transcriptional modulation of EGR-1 and Smad3/4 by GSK-3.

CREB has an important neurophysiological role, as it regulates the expression of proteins critically involved in synaptic plasticity, neuronal development, and cell fate (reviewed in Refs. 17, 18). CREB is transcriptionally activated by phosphorylation at Ser-133, a process catalyzed by a number of protein kinases and necessary for GSK-3β-mediated phosphorylation of CREB at Ser-129 (19). Several studies have demonstrated that GSK-3β negatively regulates CREB activity (20–22). Our study not only supports this notion but also suggests that GSK-3α is more robust than GSK-3β in inhibiting CRE-responsive transcriptional regulation.

EGR-1, a zinc finger transcription factor, has been implicated in diverse biological functions, including cell growth, differentiation (28, 29), and apoptosis (30–32). In cerebellar granule cells, the EGR-1 family of transcription factors plays a critical role in apoptosis by facilitating c-Jun activation (31). EGR-1 was also reported to promote p73-mediated apoptosis in N2A cells (32) and nitric oxide-induced death of SH-SY5Y cells (33). In contrast, it also has been suggested that EGR-1 confers resistance to apoptotic stimuli *in vivo* and *in vitro* (34–36). Most intriguingly, in the case of ischemia, EGR-1 is first up-regulated followed by a decline to a level below basal (36). GSK-3 activa-

tion has been implicated in the pathology of cerebral stroke (37), raising the possibility that GSK-3α and GSK-3β isoforms may be involved in the fine-tuning of EGR-1 to maintain its homeostasis after cerebral ischemia.

It has been reported that binding of TGF-β to its serine/threonine kinase receptors causes Smad2 and Smad3 to become phosphorylated. These phosphorylated Smad proteins then form a complex with Smad4 and translocate to the nucleus, where they interact with other transcription factors to activate gene expression (reviewed in Refs. 38, 39). It is worth noting that GSK-3β-specific siRNA treatment did not significantly lower Smad3/4-dependent transcriptional activity in cortical culture (Fig. 4*B*), whereas a modest inhibition of Smad3/4 transactivation in SH-SY5Y cells treated with GSK-3β siRNA mixture was observed (Fig. 4*C*). Despite this variation, substantially suppressed Smad3/4 transactivation was detected in both cell types transfected with GSK-3β R96A dominant negative mutant (Fig. 4, *B* and *C*) and thus supports the divergent effects of these two isoforms in regulating Smad3/4-dependent transcription. The differential role of GSK-3 isoforms in TGF-mediated Smad3/4 signaling is further supported by the opposite effects of GSK-3α siRNA and GSK-3β siRNA on the protein levels of *PAI-1,* which binds and inactivates tissue-type plasminogen activators (tPA). Cytoprotective and harmful effects of the tPA-plasmin system in neurological conditions have been reported (40–45). Moreover, a recent study suggests that tPA in the amygdala is involved in the anxiety-like behavior induced by stress (46). The divergent effects of GSK-3 isoforms on Smad3/4 transcriptional activity may suggest a critical role in neuropathophysiology and neuroprotection, as well as mood and anxiety regulation.

In conclusion, our study shows that selective silencing or inhibiting of two GSK-3 isoforms results in differential effectiveness or even opposite effects in regulating certain transcription factors including novel GSK-3 targets. Given that aberrant GSK-3 activity has been implicated in the pathophysiology of neurological and neuropsychiatric disorders, the development of isoform-specific inhibition or gene silencing seems to be essential for therapeutic intervention and a better understanding of the pathogenesis of these categories of diseases.

Acknowledgments—We thank Connie Chen for technical assistance and Dr. Michael Rowe and Peter Leeds of the Section for critical reading of the manuscript. We also thank the National Institutes of Health Fellows Editorial Board for superb editorial assistance.

REFERENCES

1. Bijur, G. N., and Jope, R. S. (2003) *Neuroreport* **14,** 2415–2419
2. Doble, B. W., and Woodgett, J. R. (2003) *J. Cell Sci.* **116,** 1175–1186
3. Jope, R. S., and Bijur, G. N. (2002) *Mol. Psychiatry* **7,** Suppl. 1, S35–S45
4. O'Brien, W. T., Harper, A. D., Jove, F., Woodgett, J. R., Maretto, S., Piccolo, S., and Klein, P. S. (2004) *J. Neurosci.* **24,** 6791–6798
5. Gould, T. D., Einat, H., Bhat, R., and Manji, H. K. (2004) *Int. J. Neuropsychopharmacol.* **7,** 387–390
6. Emamian, E. S., Hall, D., Birnbaum, M. J., Karayiorgou, M., and Gogos, J. A. (2004) *Nat. Genet.* **36,** 131–137
7. Woodgett, J. R. (1990) *EMBO J.* **9,** 2431–2438
8. Wang, Q. M., Fiol, C. J., DePaoli-Roach, A. A., and Roach, P. J. (1994) *J. Biol. Chem.* **269,** 14566–14574
9. Grimes, C. A., and Jope, R. S. (2001) *Prog. Neurobio.* **65,** 391–426
10. Goode, N., Hughes, K., Woodgett, J. R., and Parker, P. J. (1992) *J. Biol. Chem.* **267,** 16878–16882
11. Wang, Q. M., Park, I. K., Fiol, C. J., Roach, P. J., and DePaoli-Roach, A. A. (1994) *Biochemistry* **33,** 143–147
12. Hoeflich, K. P., Luo, J., Rubie, E. A., Tsao, M. S., Jin, O., and Woodgett, J. R. (2000) *Nature* **406,** 86–90
13. Phiel, C. J., Wilson, C. A., Lee, V. M., and Klein, P. S. (2003) *Nature* **423,** 435–439
14. Su, Y., Ryder, J., Li, B., Wu, X., Fox, N., Solenberg, P., Brune, K., Paul, S., Zhou, Y., Liu, F., and Ni, B. (2004) *Biochemistry* **43,** 6899–6908
15. Hashimoto, R., Hough, C., Nakazawa, T., Yamamoto, T., and Chuang, D. M. (2002) *J. Neurochem.* **80,** 589–597
16. Buisson, A., Lesne, S., Docagne, F., Ali, C., Nicole, O., MacKenzie, E. T., and Vivien, D. (2003) *Cell. Mol. Neurobiol.* **23,** 539–550
17. Silva, A. J., Kogan, J. H., Frankland, P. W., and Kida, S. (1998) *Annu. Rev. Neurosci.* **21,** 127–148
18. Shaywitz, A. J., and Greenberg, M. E. (1999) *Annu. Rev. Biochem.* **568,** 821–861
19. Fiol, C. J., Williams, J. S., Chou, C. H., Wang, Q. M., Roach, P. J., and Andrisani, O. M. (1994) *J. Biol. Chem.* **26,** 32187–32193
20. Bullock, B. P., and Habener, J. F. (1998) *Biochemistry* **37,** 3795–3809
21. Fang, X., Yu, S. X., Lu, Y., Bast, R. C., Jr., Woodgett, J. R., and Mills, G. B. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 11960–11965
22. Grimes, C. A., and Jope, R. S. (2001) *J. Neurochem.* **78,** 1219–1232
23. Deng, J., Xia, W., Miller, S. A., Wen, Y., Wang, H. Y., and Hung, M. C. (2004) *Mol. Carcinog.* **39,** 139–146
24. Bournat, J. C., Brown, A. M., and Soler, A. P. (2000) *J. Neurosci. Res.* **61,** 21–32
25. Qin, J. Z., Chaturvedi, V., Denning, M. F., Choubey, D., Diaz, M. O., and Nickoloff, B. J. (1999) *J. Biol. Chem.* **274,** 37957–37964
26. Schneider, A., Martin-Villalba, A., Weih, F., Vogel, J., Wirth, T., and Schwaninger, M. (1999) *Nat. Med.* **5,** 554–559
27. Blom, N., Sicheritz-Ponten, T., Gupta, R., Gammeltoft, S., and Brunak, S. (2004) *Proteomics* **4,** 1633–1649
28. Harada, T., Morooka, T., Ogawa, S., and Nishida, E. (2001) *Nat. Cell Biol.* **3,** 453–459
29. Lee, J. H., and Kim, K. T. (2004) *J. Neurochem.* **91,** 634–647
30. Liu, C., Rangnekar, V. M., Adamson, E., and Mercola, D. (1998) *Cancer Gene Ther.* **5,** 3–28
31. Levkovitz, Y., and Baraban, J. M. (2001) *J. Neurosci.* **21,** 5893–5901
32. Pignatelli, M., Luna-Medina, R., Perez-Rendon, A., Santos, A., and Perez-Castillo, A. (2003) *Biochem. J.* **373,** 739–746
33. Cibelli, G., Policastro, V., Rossler, O. G., and Thiel, G. (2002) *J. Neurosci. Res.* **67,** 450–460
34. Hallahan, D. E., Dunphy, E., Virudachalam, S., Sukhatme, V. P., Kufe, D. W., and Weichselbaum, R. R. (1995) *J. Biol. Chem.* **270,** 30303–30309
35. Huang, R. P., Fan, Y., deBelle, I., Ni, Z., Matheny, W., and Adamson, E. D. (1998) *Cell Death Differ.* **5,** 96–106
36. Carmel, J. B., Kakinohana, O., Mestril, R., Young, W., Marsala, M., and Hart, R. P. (2004) *Exp. Neurol.* **185,** 81–96
37. Sasaki, C., Hayashi, T., Zhang, W. R., Warita, H., Manabe, Y., Sakai, K., and Abe, K. (2001) *Neurol. Res.* **23,** 588–592
38. Shi, Y., and Massague, J. (2003) *Cell* **113,** 675–700
39. Derynck, R., and Zhang, Y. E. (2003) *Nature* **425,** 577–584
40. Tsirka, S. E., Rogove, A. D., and Strickland, S. (1996) *Nature* **384,** 123–124
41. Tabrizi, P., Wang, L., Seeds, N., McComb, J. G., Yamada, S., Griffin, J. H., Carmeliet, P., Weiss, M. H., and Zlokovic, B. V. (1999) *Arterioscler. Thromb. Vasc. Biol.* **19,** 2801–2806
42. Soeda, S., Oda, M., Ochiai, T., and Shimeno, H. (2001) *Brain Res. Mol. Brain Res.* **91,** 96–103
43. Docagne, F., Nicole, O., Gabriel, C., Fernandez-Monreal, M., Lesne, S., Ali, C., Plawinski, L., Carmeliet, P., MacKenzie, E. T., Buisson, A., and Vivien, D. (2002) *Mol. Cell. Neurosci.* **21,** 634–644
44. Akassoglou, K., Yu, W. M., Akpinar, P., and Strickland, S. (2002) *Neuron* **33,** 861–875
45. Melchor, J. P., Pawlak, R., and Strickland, S. (2003) *J. Neurosci.* **23,** 8867–8871
46. Pawlak, R., Magarinos, A. M., Melchor, J., McEwen, B., and Strickland, S. (2003) *Nat. Neurosci.* **6,** 168–174