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# Phosphatidylinositol 3-Kinase and Glycogen Synthase Kinase 3 Regulate Estrogen Receptor-Mediated Transcription in Neuronal Cells

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**In addition to 17β-estradiol binding, estrogen receptor (ER) transcriptional activity could be controlled by intracellular kinase signaling pathways activated by growth factors. In this report we present evidence suggesting that glycogen synthase kinase 3 (GSK3), an effector kinase of the phosphatidylinositol 3-kinase (PI3K) pathway, may affect ERα activity in N2a neuroblastoma cells. LiCl, sodium valproate, and SB415286, three inhibitors of GSK3, dose-dependently blocked ERα-mediated transcription. In contrast, overexpression of wild-type GSK3, but not of a mutant inactive form, increased ER-dependent gene expression. Pharmacological or genetic inhibition of the PI3K/Akt pathway, whose activity is inversely correlated with that of GSK3, increased ERα-mediated transcription, and this effect was blocked by GSK3 inhibitors. As in other cell types, IGF-I increased ERα activity in absence of estradiol by a mechanism independent of PI3K. In contrast, IGF-I decreased ERα activity in the presence of estradiol, and this effect was mediated by PI3K. We also observed a regulated interaction between β-catenin, one of the main GSK3 nuclear targets, and ERα. Transfection with a nondegradable mutant of β-catenin blocked the increase in ERα transcriptional activity induced by the PI3K inhibitor wortmannin, suggesting a role for β-catenin in estrogen signaling. In addition, we investigated the regulation of ER protein levels as a potential mechanism for its regulation by the PI3K/GSK3 pathway; GSK3 blockade increased ERα protein stability, whereas PI3K inhibition decreased it. In summary, our findings suggest that ER-dependent gene expression in N2a cells is controlled by the PI3K/Akt/GSK3 signaling pathway. (*Endocrinology* 147: 3027–3039, 2006)**

THE OVARIAN HORMONE 17β-estradiol (βE2) exerts profound effects on the physiology and pathology of the central nervous system (CNS) (1). In the brain, βE2 is able to regulate the expression of a wide subset of genes and the activity of multiple signaling systems. The main biological actions of βE2 are mediated by its binding to its cellular receptors that belong to the superfamily of nuclear receptors (2). The two identified subtypes of estrogen receptors (ERs), ERα and ERβ, are abundantly expressed in the brain (3). These are transcription factors that upon ligand binding, directly bind to DNA in the promoter regions of many genes in which an estrogen response element (ERE) exists. ERs can be divided into six (A–F) functional and physical domains, which encode the regions required for hormone binding (E), nuclear localization (D), or DNA binding (C; Fig. 1A). ERs have two separate transactivation domains: activation function 1 (AF1) localized in domain A/B, and AF2 localized in domain E (4). The ability of ERs to induce changes in gene expression is mediated by their interaction with members of the mRNA synthesis machinery, which include coactivators and corepressors (5).

In addition to βE2 binding, ER transcriptional activity can be regulated by ligand-independent mechanisms (6). Intracellular kinase signaling pathways, activated by extracellular growth or trophic factors, regulate the ability of ERs to promote changes in gene expression. IGF-I is one of the extracellular regulators of these kinase pathways that has been shown to promote ERE-dependent transcription (7, 8). In addition, IGF-I has been shown to have a wide interdependence with estrogen in the promotion of its multiple effects, in particular in the CNS (9–13). The functional relevance of this cross-talk between IGF-I and estrogen in the CNS is quite well established, but the mechanisms are only beginning to be understood (14).

Two signaling pathways regulated by IGF-I and other extracellular factors are the MAPK and the phosphatidylinositol 3-kinase (PI3K) pathways (15). IGF-I, epidermal growth factor, nerve growth factor, and brain-derived neurotropic factor activate both intracellular cascades by binding to their cognate membrane receptors. Different members of MAPK and PI3K signaling systems, *i.e.* Akt and ERK, phosphorylate and positively regulate the transactivation properties of ERα (16, 17). The AF1 in the N-terminal domain of ERα is essential for growth factor activation of its transcriptional activity (16). However, direct phosphorylation is not the only way in which intracellular kinases modify ER activity. ER coactivators of the p160 family, such as steroid receptor coactivator 1, have also been shown to be targets of the MAPK/ERK pathway (18).

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Abbreviations: AF, Activation function; C/D, charcoal and dextran treated; CMV, cytomegalovirus; CNS, central nervous system; DPN, 2,3-bis(4-hydroxyphenyl) proprionitrile; βE2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; FCS, fetal calf serum; βGal, β-galactosidase; GSK3, glycogen synthase kinase 3; IGF-IR, IGF-I receptor; pHEGO, human ERα expression plasmid; PI3K, phosphatidylinositol 3-kinase; PPT, propylpyrazole triol; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid (RARE) response element; SEAP, secreted alkaline phosphatase; SH-6, Akt inhibitor III; wt, wild type.

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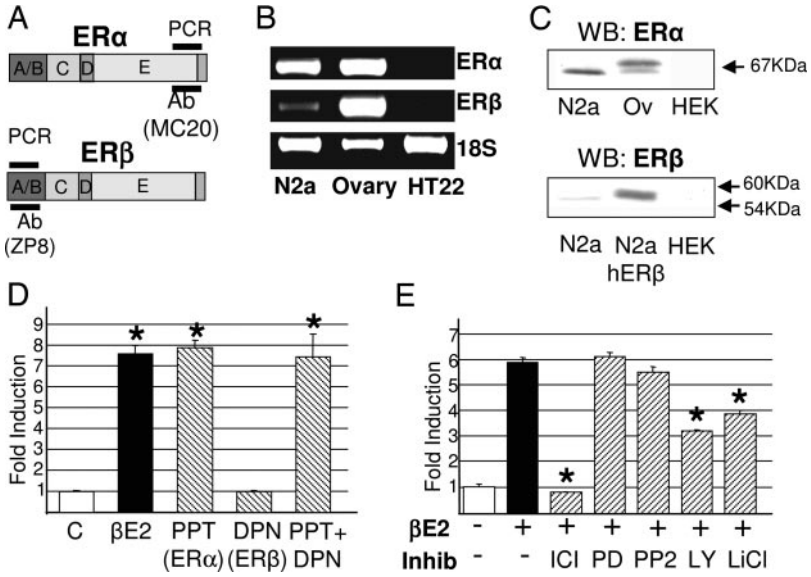


FIG. 1. ERα expressed by N2a cells is controlled by intracellular kinases. A, Mapping of the primers (PCR) and antibodies (Ab) used for PCR and Western blot analyses, respectively, of both subtypes of ERs (ERα and ERβ) in N2a cells. B, cDNAs from N2a cells, mouse ovary, and HT22 cells were amplified using pairs of specific primers for ERα, ERβ, and a constitutive gene (18S RNA). Transcript for ERα is observed in N2a cells and ovary. Transcript for ERβ is clearly seen in the ovary. In N2a cells, the level of ERβ transcript is very low, but detectable. ER transcripts are not detected in HT22 cells. C, Protein extracts from N2a cells (20 μg) were tested for the presence of ERα and ERβ using specific antibodies and compared with those in cell lysates obtained from HEK293 cells (HEK; 20 μg), rat ovary (Ov; 50 μg), or human ERβ-overexpressing N2a cells (N2a hERβ; 1 μg). ERα was clearly detected in N2a cells and ovary extracts, but not in HEK293 cells. Recombinant ERβ was detected in ERβ-transfected N2a cells, whereas endogenous ERβ was only detected in nontransfected N2a cells (and not in transfected cells due to the different amount of protein loaded in each case). D, After transfection with the reporter plasmids (pTA-ERE-SEAP and pCMV-βGal), cells were incubated with 1 nM βE2 (ERα and ERβ agonist), 10 nM PPT (ERα agonist), 10 nM DPN (ERβ agonist), and 10 nM PPT plus 10 nM DPN. ER-dependent gene expression was quantified by measuring SEAP activity and normalized for transfection efficiency. Data are expressed as the mean ± SEM fold induction detected with each compound compared with that in control cells (C; vehicle treated). βE2 and the ERα-specific agonist PPT increased the activity of the SEAP reporter gene, whereas DPN had no effect. *, $P < 0.05$ *vs.* control values. E, The βE2 induction of ER dependent reporter activity was quantified in the presence of the estrogen receptor antagonist ICI182780 (ICI; 100 nM) or inhibitors of intracellular kinases, PD98059 (PD; 25 μM), PP2 (1 μM), LY294002 (LY; 25 μM), and LiCl (10 mM), that were added to the culture medium 45 min before βE2 treatment. Data are expressed as described in D. The ER antagonist ICI182780 completely blocked the expression of the reporter gene induced by βE2. The MAPK inhibitor PD98059 and the Src inhibitor PP2 had no effect. In contrast, the PI3K inhibitor LY294002 and the GSK3 inhibitor LiCl significantly decreased the expression of the reporter gene induced by βE2. *, $P < 0.05$ *vs.* βE2 alone.

Glycogen synthase kinase 3 (GSK3) is a component of the PI3K signaling pathway. It is a serine/threonine kinase widely expressed in mammalian tissues that has two isoforms, GSK3α and GSK3β (19). It has critical roles during development, and in adult organisms regulates glycogen and lipid metabolism, cytoskeletal dynamics, and apoptosis (19). GSK3, unlike most kinases, has a high level of activity in resting cells that is negatively regulated by phosphorylation of the serine residue in the N-terminal region of the molecule (serine 11 for GSK3α and serine 9 for GSK3β). This phosphorylation is effected, upon hormonal or growth factor stimulation, by intracellular kinases such as Akt, protein kinase A, and p70S6 kinase (20). Despite the specificity that its name suggests, GSK3 has a wide variety of targets in intracellular signaling systems, including many transcription factors and nuclear proteins such as activation protein 1, cAMP response element-binding protein, nuclear factor-κB, and β-catenin (19). Some of these proteins are directly phosphorylated by GSK3, such as β-catenin or c-Jun, a component of activation protein 1 transcription factor. Other molecules, such as nuclear factor-κB, are not direct targets of GSK3 phosphorylation, but they are regulated by changes in its activity (21). In the present study using N2a neuroblastoma cells as a model, we investigated the role of the PI3K/Akt/GSK3 signaling pathway in the control of estrogen receptor-mediated transcription in neuronal cells.

## Materials and Methods

### *Cell culture*

Neuro-2a (N2a) neuroblastoma cells (American Type Culture Collection, Manassas, VA; CCL-131) were gifts from Dr. M. A. Arevalo (Instituto Cajal, Madrid, Spain). They were cultured in DMEM/Ham's F-12 medium supplemented with 10% fetal calf serum (FCS; Invitrogen Life Technologies, Inc., Barcelona, Spain). For experimental procedures, they were plated in medium supplemented with 10% charcoal- and dextran-treated FCS (C/D FCS; HyClone, Logan, UT) in six- and 12-well plates (Falcon, BD Biosciences, Madrid, Spain). The same procedure was used to culture HEK293 and HT22 cell lines. The cells used in the experiments presented in this report were cultured for no more than 10 passages.

### *Materials and expression plasmids*

The inhibitors LY294002, PD98059, MG132, SH-6, and wortmannin were purchased from Calbiochem (Merck, Nottingham, UK). SB415286 was obtained from Tocris (Avonmouth, UK). βE2, lithium chloride, and valproic acid (sodium salt) were obtained from Sigma-Aldrich Corp. (St. Louis, MO). ICI182780 was obtained from Zeneca Pharmaceuticals (Cheshire, UK). The subtype-specific estrogen receptor agonists propylpyrazole triol (PPT) and 2,3-bis(4-hydroxyphenyl) proprionitrile (DPN) were purchased from Tocris and were predissolved, as was βE2, in

dimethylsulfoxide. Control cultures received similar amounts of solvent (vehicle). IGF-I was purchased from Gro-Pep (Adelaide, Australia). The polyclonal antibodies against mouse ERα (MC20) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ERβ antibody (ZP8) was purchased from Zymed Laboratories (Invitrogen Life Technologies, Inc., Barcelona, Spain). The monoclonal antibody against human ERα (AER611) used for Western blotting and immunoprecipitation protocols was purchased from NeoMarkers (Fremont, CA). The anti-GSK3 and β-catenin monoclonal antibodies were purchased from BD Transduction Laboratories (Madrid, Spain). Anti-p85 antiserum was a gift from Dr. S. Pons (Instituto de Investigaciones Biomédicas de Barcelona, Barcelona, Spain). M2 antibody, raised against the Flag tag sequence, was purchased from Sigma-Aldrich Corp. βIII tubulin monoclonal antibody was purchased from Promega Corp. (Barcelona, Spain), and the antibodies against phosphorylated forms of GSK3 and Akt were obtained from Cell Signaling Technology (Beverly, MA).

Constructs of the secreted alkaline phosphatase (SEAP) reporter gene controlled by a minimal promoter and ERE or retinoic acid (RA) response element (RARE; pERE-SEAP and pRARE-SEAP, respectively) were purchased from BD Biosciences. The expression plasmids for rat GSK3β (22) and β-catenin (23) forms were gifts from Dr. F. Wandosell (Centro de Biología Molecular, Madrid, Spain). The human ERβ expression plasmid was obtained from Dr. P. Auricchio (Dipartimento di Patologia Generale, Universita di Napoli, Naples, Italy); pHEGO, encoding human ERα, was a gift from Dr. P. Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). Both forms of the murine p85 subunit of PI3K, wild-type p85 (p85wt) and p85Δ110 (both fused to a Flag epitope) (24), and of human IGF-I receptor (IGF-IR), the wt form (IGFIRwt) and a mutated form (IGFIR-KR) (25), were gifts from Dr. S. Pons (Barcelona, Spain).

### *RT-PCR*

Total RNA from N2a cells and mouse ovary were isolated with TRIzol reagent (Invitrogen Life Technologies, Inc.). Two micrograms of total RNA were used for a retro transcription reaction using Moloney's murine leukemia virus retrotranscriptase (Promega Corp.). The products of these reactions were used as substrates for PCR, using pairs of primers specific for murine ERα (accession no. M38651) or ERβ (accession no. AJ000220) (26). The sequences of these primers were as follows: ERα sense, CAAAGCTGGCCTGACTCTGC; ERα antisense, CCTCTGCTTCCGGGGGTATGTA; ERβ sense, GTCCTGCTGTGATGAACTAC; and ERβ antisense, CCCTCTTGGTGCTTGGACT. For both ERs, the amplified region spanned different exons that corresponded to the C-terminal region of the receptor in the case of ERα (nucleotides 1682–1981) and to the N-terminal region (nucleotides 10–282) in the case of ERβ (Fig. 1A). The primers used for amplification of 18S ribosomal RNA were purchased from Ambion, Inc. (Austin, TX), and produced a fragment of 488 nucleotides. The products of PCR were resolved in 1.5% agarose gels and stained with ethidium bromide.

### *Transient expression and reporter gene experiments*

We measured the estrogen dose- and time-dependent responses of ERE reporter constructs to identify the best conditions for studying ER-mediated transcription in N2a cells (data not shown). We cotransfected a plasmid expressing the β-galactosidase gene under the control of a constitutive promoter [cytomegalovirus-β-galactosidase promoter (pCMV-βGal)] to normalize the values of SEAP activity to transfection efficiency and detect changes in basic transcriptional machinery. For reporter gene experiments, cells were plated in 12-well plates with 10% C/D FCS 24 h before overnight transfection with FuGene (Roche, Mannheim, Germany). Normally, the transfection mixture included 100–200 ng pERE-SEAP (or pRARE-SEAP) and 100 ng pCMV-βGal. When needed, overexpression plasmids for GSK3, IGF-IR, p85, or β-catenin or the empty vector (pcDNA3; Invitrogen Life Technologies, Inc.) were added to this mixture (200 ng/well). After transfection, the medium was changed to 2% C/D FCS; 1 d later, cells were subjected to the experimental treatments for 24 h. Culture supernatants were collected for determining SEAP activity using a luminescent assay (BD Biosciences), and cell lysates were assayed for βGal activity (27). Data are presented as the fold induction of reporter activity in each experiment. For Western blotting and immunoprecipitation purposes, cells were cultured in 35-mm diameter wells in medium containing 10% C/D FCS. When needed, they were transfected with overexpressing plasmids (0.2 μg/well). All treatments were applied after incubation for at least 24 h in culture medium with reduced serum (2% C/D FCS).

### *Immunoprecipitation and Western blot experiments*

Cells were washed once with PBS and lysed in a buffer containing 150 mM NaCl, 20 mM Tris HCl, 10% glycerol, 5 mM EDTA, and 1% Nonidet P-40 (Roche) supplemented with protease and phosphatase inhibitors (50 μg/ml phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, 25 μg/ml leupeptin, and 100 nM orthovanadate; all from Sigma-Aldrich Corp.). Homogenates were briefly sonicated, solubilized for 30 min on ice, and centrifuged at 21,000 × *g* for 10 min. The protein content of the supernatant was measured with a modified Bradford assay (Bio-Rad Laboratories, Munich, Germany). Aliquots containing 300 μg protein were subjected to immunoprecipitation using specific antibodies. The immune complexes were adsorbed and precipitated using preequilibrated protein A-Sepharose beads (Amersham Biosciences, Little Chalfont, UK), washed three times with lysis buffer, and denatured by boiling for 5 min in sample buffer [13 mM Tris (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate, 0.1 M dithiothreitol, and 0.002% bromophenol blue]. For analysis of ERα stability, cells were lysed in a buffer containing 10 mM Tris (pH 7), 10 mM NaCl, 3 mM $MgCl_2$, 0.05% Nonidet P-40, and 1 mM EGTA, supplemented with protease and phosphatase inhibitors. Cell lysates were centrifuged at 1000 × *g* for 10 min at 4 C to obtain the cytosolic fraction. The immunoprecipitates, cellular lysates, or fractions (10 μg) were resolved by SDS-PAGE in a Mini-Protean system (Bio-Rad Laboratories) and transferred to nitrocellulose membranes where specific proteins where immunodetected using an enhanced chemiluminescence system (ECL, Amersham Biosciences). Films were analyzed using Image J software (developed at the National Institutes of Health and available on the internet at http://rsb.info.nih.gov/ij/). The density of each band was normalized to its respective loading control and represented as a percentage of the control value (cultures treated with vehicle). When needed, membranes were stripped using a stripping buffer provided by Chemicon International (Temecula, CA).

### *Immunofluorescence*

For immunofluorescence analysis, cells were grown on gelatin-treated coverslips. After washing twice with PBS, they were fixed with 4% paraformaldehyde for 15 min at room temperature, washed again with PBS, and incubated overnight at −4 C with the primary antibodies [anti-ERα and AER611 (NeoMarkers, Fremont, CA), diluted 1:250; anti-GSK3β and anti-β-catenin (BD Transduction Laboratories), diluted 1:500], a solution of 0.1% Triton X-100, 0.1% BSA, and 3% normal goat serum. The coverslips were washed extensively with PBS and incubated with secondary antibodies conjugated with Alexa Red 568 or Alexa Green 488 (both diluted 1:1000; Molecular Probes, Eugene, OR) for 2 h at room temperature. They were mounted onto glass slides using Mobiol-based mounting medium and examined using a confocal microscope (Leica, Heidelberg, Germany).

### *Statistical analysis*

Reporter experiments were repeated at least three times. For Western blots, at least three different experiments were quantitatively analyzed. One-way ANOVA followed by *post hoc* analysis with the Bonferroni test, unless otherwise specified, were used to determine statistically significant differences among three or more groups. $P < 0.05$ was adopted as the threshold of statistical significance. Data are represented as the mean ± SEM.

## Results

### *Activity of functional ERα expressed in N2a cells is controlled by inhibitors of intracellular kinases*

We performed RT-PCR, Western blot, and reporter gene-based analysis of N2a cells to assess whether they express functional ERs. Using primers directed against different exons of the C-terminal region of ERα and the N-terminal

region of ERβ, we detected transcripts for both subtypes of ER (Fig. 1, A and B) in N2a cells and ovary, but not in HT22 cells. The level of ERβ transcript was very low, but detectable, in N2a cells. The amplified fragments had the correct size, as expected by analysis of the published sequences for both murine receptors (see *Materials and Methods*). For Western blotting experiments, we used two different antibodies, one directed against ERα (MC20) and one directed against ERβ (Z8P). We used HEK293 cells as a negative control for the antibodies, because these cells do not express ERs. We also used mouse ovary as a positive control for MC20 antibody and N2a cells overexpressing human ERβ as a positive control for the Z8P antibody directed against ERβ. In N2a cells, the MC20 antibody detected a band of approximately 67 kDa, the expected molecular mass for ERα (Fig. 1C). This band was also observed in mouse ovary together with another band of slightly higher molecular mass. The ERβ antibody detected both recombinant human ERβ and the endogenous protein in N2a cells (Fig. 1C). ERs were not detected in HEK293 cells (Fig. 1C).

We used an estrogen-sensitive reporter gene (pTA-ERE-SEAP) to test the functionality of these endogenous receptors. We compared the response evoked by βE2, the natural ligand for both subtypes of ERs, with the activation produced by two compounds that bind to and activate only one subtype of receptor: PPT, a selective ligand for ERα (28), and DPN, a selective ligand for ERβ (29). Cells were stimulated with 1 nM βE2, 10 nM PPT, 10 nM DPN, or 10 nM PPT plus 10 nM DPN. PPT, the ERα-specific agonist, increased the activity of the SEAP reporter gene to a similar extent as βE2, whereas DPN did not have any effect (Fig. 1D). Simultaneous treatment with both ligands, PPT and DPN, activated the reporter to levels similar to those reached after βE2 treatment.

Having established the functionality of the ERs expressed by N2a cells, we screened several commonly used kinase inhibitors for their abilities to modulate this estrogen-responsive gene reporter assay. The inhibitors tested were PD98059 for ERKs, PP2 for Src kinase, LY294002 for PI3K, and lithium chloride (LiCl) for GSK3. We compared the effects of these inhibitors with that of the blockade exerted by ICI182780, a pure antagonist of classic ERs. N2a cells were pretreated with these kinase inhibitors or ICI182780 for 45 min, then stimulated with 1 nM βE2. SEAP activity was determined 24 h later. As shown in Fig. 1E, ICI182780 completely blocked βE2-induced expression of the reporter gene, whereas MAPK and Src inhibitors had no effect. Both LY294002 and LiCl significantly decreased βE2-induced expression of the reporter gene (by 54% and 40%, respectively). Although LY294002 has direct antiestrogenic activity that could be responsible for the inhibitory effect seen in our experiments (30), these results suggest a possible role of PI3K/GSK3 in the control of ER-mediated transcription.

### *Effects of GSK3 inhibitors and GSK3 overexpression on the activity of an estrogen-sensitive reporter construct*

To investigate the role of GSK3 in the regulation of ER-mediated transcription in N2a cells, we assessed the effects of different concentrations of LiCl, sodium valproate, and SB415286, three pharmacological inhibitors of GSK3 (31–33), in the estrogen-responsive gene reporter assay. The three inhibitors tested resulted in a decrease in ER-mediated transcription in a dose-dependent manner (Fig. 2A). The inhib-

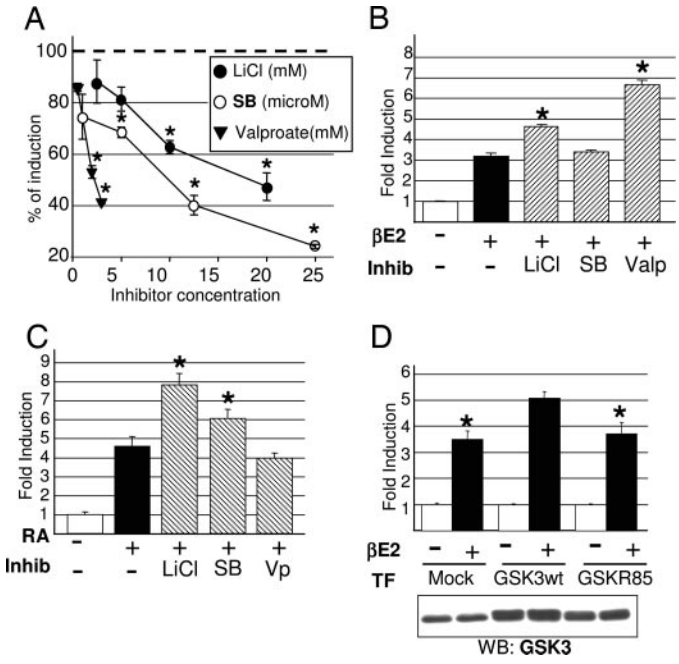


FIG. 2. GSK3 activity specifically controls ER-dependent gene expression in N2a cells. A, N2a cells were transfected with the ERE reporter plasmid and treated with different concentrations of GSK3 inhibitors 45 min before βE2 (1 nM) treatment. The concentrations used were 2.5, 5, 10, and 20 mM for LiCl; 0.5, 2, and 3 mM for sodium valproate; and 1, 5, 12.5, and 25 μM for SB415286 (SB). The plots represent the mean ± SEM percentage of the total response (100%; elicited by βE2 alone). The three inhibitors tested resulted in a decrease in ER-mediated transcription in a dose-dependent manner. *, $P < 0.05$ *vs.* βE2 alone. B, HEK293 cells were transfected with ERα and the ERE reporter plasmid. Cells were treated with the GSK3 inhibitors (Inhib) LiCl (10 mM), SB415286 (SB; 25 μM), and sodium valproate (Valp; 2 mM) 45 min before the addition of 1 nM βE2. ERα-dependent gene expression was measured 24 h latter by quantification of SEAP activity. Data are normalized to transfection efficiency and are presented as the mean ± SEM. In contrast to their inhibitory effect on N2a cells, the GSK3 inhibitors LiCl and valproate increased ER-mediated transcription in HEK293 cells, whereas SB415286 was without effect. *, $P < 0.05$ *vs.* βE2 alone. C, N2a cells were transfected with an RARE construct (pTA-RARE-SEAP). The GSK3 inhibitors (Inhib) LiCl (10 mM), SB415286 (SB; 25 μM), and valproate (Valp; 2 mM) were added 45 min before treatment with 1 μM RA. Activation of the reporter gene was measured 24 h latter and is represented, normalized, as the mean ± SEM. In contrast to their inhibitory effect on ER-mediated transcription, the GSK3 inhibitors LiCl and SB415286 enhanced RA-mediated transcription, whereas valproate was without effect. *, $P < 0.05$ *vs.* RA alone. D, Cells were cotransfected with pTA-ERE-SEAP and three different plasmids (TF): an empty vector (Mock), the wt rat GSK3 (GSK3wt), or a mutant, kinase-inactive GSK3 (GSKR85). Twenty-four hours after transfection, cells were treated with βE2 (1 nM) or its vehicle for an additional 24 h. SEAP activity was then quantified and normalized to transfection efficiency. The levels of GSK3 in each situation, as evaluated by Western blotting, are shown below. The results are expressed as the mean fold induction in each situation ± SEM. Overexpression of GSK3wt resulted in an increase in ER-mediated transcription. This effect was not observed when the mutated inactive form of GSK3 (GSKR85) was overexpressed. *, $P < 0.05$ *vs.* βE2 response in the GSK3wt-transfected group.

itory effects of SB415286, LiCl, and valproate were also observed when cells were stimulated with PPT, the ERα-specific agonist (not shown). To test whether GSK3 inhibition affects ERα activity in other cell types, we performed experiments using HEK293 cells. Cells were transfected with ERα and pTA-ERE-SEAP and treated with βE2 in the presence or absence of GSK3 inhibitors. None of the GSK3 inhibitors tested produced a decrease in ERα-mediated transcription in HEK293 cells (Fig. 2B). LiCl (10 mM) and valproate (3 mM) increased the response of ERα in HEK293 cells by 62% and 150%, respectively, whereas SB415285 showed no effect (Fig. 2B). In addition, we investigated whether the effect of GSK3 inhibition was similar to those of other members of the superfamily of nuclear receptors to which ERα belongs. We tested the effects of LiCl, valproate, and SB415286 on the activity of RA receptor (RAR) in N2a cells. For this experiment, N2a cells were transfected with a reporter construct in which SEAP reporter gene was under the control of RARE. N2a express functional RAR whose activity is enhanced by RA treatment. The GSK3 inhibitors, LiCl and SB415286, increased the reporter response by 91% and 42%, respectively (Fig. 2C). In contrast, valproate showed no effect on RAR-mediated gene expression (Fig. 2C).

Because some GSK3 inhibitors used in this study may have other targets, we decided to use genetic tools to demonstrate GSK3 control of ER-mediated transcription in N2a cells. We overexpressed two different forms of the kinase, the wt form (GSK3βwt) and a mutated, kinase-inactive form (GSK3βR85) (22). Although the levels achieved were not very high compared with the control (Fig. 2D), the overexpression of GSK3wt resulted in a significant increase (~40%) in the response of the reporter construct to the hormone. In contrast, the enhancement of ERα-mediated transcription was not observed when the mutated, kinase-inactive form of GSK3 (GSK3βR85) was transfected (Fig. 2D).

### *Effects of Akt and PI3K activities on ER-mediated transcription in N2a cells*

It is well established that one of the main signaling cascades modulating GSK3 activity in mammalian cells is the PI3K/Akt pathway (34–37). Therefore, we decided to investigate the role of GSK3 in PI3K control of ER-mediated transcription in N2a cells. Because, as mentioned previously, LY294002 has potential antiestrogenic effects due to its direct inhibitory binding to ERs (30), we tested the effect of another pharmacological PI3K inhibitor, wortmannin. Wortmannin produced a decrease in the serine 9 phosphorylation of GSK3 (Fig. 3A) and induced a dose-dependent increase in ER-mediated transcription (Fig. 3B). The two GSK3 inhibitors tested, LiCl (Fig. 3B) and SB415286 (not shown), blocked the effect of wortmannin on ER activity, suggesting that GSK3 mediates the effect of PI3K on ER-mediated transcription. In addition, we performed experiments blocking the PI3K pathway using a dominant-negative form of p85, the regulatory subunit of this kinase. We used p85Δ110, a truncated form of p85 that lacks a small region implicated in the interaction with p110, the catalytic subunit of PI3K (24). The expression of wt and truncated forms of p85 was confirmed by Western blotting using an antibody against the Flag tag carried by both proteins (Fig. 3C). In both cases, the levels of expression achieved were well over those of endogenous p85, as reveled by Western blotting with the p85 antibody, which recognizes endogenous and both transfected forms (Fig. 3C). The overexpression of the mutated form resulted in a significant decrease in the serine 9 phosphorylation of GSK3 (Fig. 3C) and a 45% increase in ER-mediated transcription compared with cells overexpressing the wild-type form of p85 (Fig. 3D). We consider it improbable, although it cannot be completely excluded, that small differences in the expression of the wild-type and truncated forms of p85 (Fig. 3C) may in part cause the observed differences in ER activation. However, the increase in ER-mediated transcription in N2a cells caused by the truncated form of p85 was no longer present in the presence of the GSK3 inhibitors LiCl (Fig. 3D) and SB415286 (not shown).

The specific Akt inhibitor III (SH-6) (38), produced a decrease in the serine 9 inhibitory phosphorylation of GSK3 (Fig. 3E). In addition, SH-6 induced a dose-dependent increase in ER-mediated transcription (Fig. 3F). In this experiment we tested the effects of the GSK3 inhibitors LiCl (Fig. 3F) and SB415286 (not shown). Both inhibitors blocked the effect of SH-6 on ER activity, suggesting that GSK3 mediates the effect of Akt on ER-mediated transcription.

The activity of GSK3 is finely regulated in mammalian cells through many signaling pathways. In the brain, the phosphorylation of serine 9 of GSK3β by Akt kinase is one of the main mechanisms by which some extracellular factors, such as IGF-I and nerve growth factor (34–36), reduce the constitutive activity of GSK3β. In the next set of experiments we tested the potential effects of extracellular regulators of PI3K/Akt pathway on the transcriptional activity of ERs.

We first analyzed the effect of βE2 on Akt and GSK3 phosphorylation. As shown in Fig. 4A, βE2 did not affect the phosphorylation levels of Akt serine 473 or GSK3β serine 9. Moreover, βE2 did not affect the wortmannin-induced decrease in the phosphorylation levels of both kinases. We then tested the ability of endogenous IGF-IR to regulate Akt and GSK3 phosphorylation and ER-mediated transcription in N2a cells. IGF-I treatment dose-dependently increased the phosphorylation of Akt on serine 473 in a wortmannin-sensitive manner (Fig. 4B). A similar effect was observed when we analyzed the phosphorylation of serine 9 of GSK3β (Fig. 4B).

To assess whether IGF-I affects ER-mediated transcription, N2a cells were transfected with the reporter plasmid and treated with βE2 and IGF-I alone or in combination. Treatment with IGF-I induced a small, but significant, activation of the reporter construct (~20% of the βE2 response) that was not affected by wortmannin treatment (Fig. 4C). In contrast, the addition of both hormones, βE2 and IGF-I, produced a smaller response than that observed in the presence of βE2 alone (Fig. 4C). This inhibitory effect was no longer detected in the presence of wortmannin (Fig. 4C).

Because N2a cells express low levels of IGF-IR (Fig. 4D), we tested the effect of the overexpression of two different forms of IGF-IR. The overexpression of IGFIRwt in N2a cells had an inhibitory effect on βE2-induced, ER-mediated transcription. This effect was not detected when a kinase-dead form of this receptor (IGFIR-KR) was transfected in N2a cells (Fig. 4D).

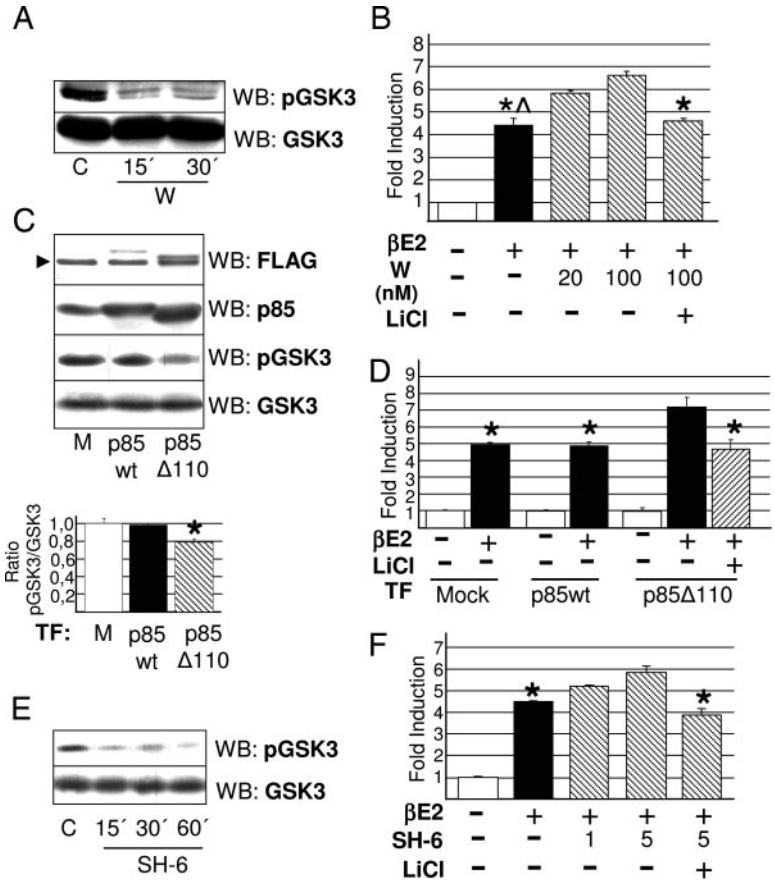


FIG. 3. PI3K regulates ER-dependent transcription through GSK3 in N2a cells. A, N2a cells were cultured in medium containing 2% C/D FCS for 24 h and treated with vehicle (C) or the PI3K inhibitor wortmannin (W; 100 nM) for 15 or 30 min. The phosphorylation of GSK3$\beta$ in serine 9 was analyzed in cell lysates by Western blotting using a phosphospecific antibody (pGSK3; *upper gel*). Total GSK3$\beta$ was used as a control (GSK3; *lower gel*). Treatment with wortmannin produced a decrease in the phosphorylation of GSK3. B, N2a cells were transfected with pTA-ERE-SEAP and 24 h latter treated with the PI3K inhibitor wortmannin (W; 20 or 100 nM) in the presence or absence of 10 mM LiCl (30-min pretreatment). Forty-five minutes later, $\beta$E2 (1 nM) was added to the medium for an additional 24 h. The graph shows the induction of reporter activity in each situation normalized to transfection efficiency. Data are presented as the mean ± SEM. Wortmannin induced a dose-dependent increase in ER-mediated transcription. The GSK3 inhibitor LiCl blocked the effect of wortmannin. *, $P < 0.05$ *vs.* $\beta$E2 plus 100 nM W; ∧, $P < 0.05$ *vs.* $\beta$E2 plus 20 nM W. C, Cells were cotransfected with pTA-ERE-SEAP and the expression plasmids for two different forms of p85 subunit of PI3K: the wt sequence (p85 wt) and the mutant negative form (p85Δ110). A group of cells was transfected with the empty vector (M). To confirm overexpression of the wt and mutated forms of p85, cell lysates were analyzed using an antibody against the Flag antibody fused to both forms of p85 (FLAG; *upper gel*) or an antibody against p85 (p85; *second gel*). In addition, the phosphorylation of GSK3$\beta$ in serine 9 was analyzed using a phosphospecific antibody (pGSK3; *third gel*). Total GSK3$\beta$ was used as a control (GSK3; *lower gel*). The *arrowhead* marks an unspecific band detected by the FLAG antibody in N2a cells. M, Cells transfected with an empty vector; p85 wt, cells transfected with the expression plasmid for the wt form of p85; p85 Δ110, cells transfected with the expression plasmid for the mutated form of p85. *Lower panel*, pGSK3/GSK3 ratio. Overexpression of the mutated form of p85 resulted in a significant decrease in the phosphorylation of GSK3. *, $P < 0.05$ *vs.* mock-transfected cells (M). D, Cells were cotransfected with pTA-ERE-SEAP and three different plasmids: an empty vector (Mock), the wt form (p85wt) of p85, or the mutated form (p85Δ110) of p85. Twenty-four hours later, cells were pretreated with LiCl for 45 min, then with $\beta$E2 (1 nM) for an additional 24 h. The graph shows the induction of reporter activity in each situation normalized to transfection efficiency. Data are presented as the mean ± SEM. Overexpression of the mutated form of p85 (p85Δ110) resulted in a significant increase in ER-mediated transcription compared with cells overexpressing the wt form. The GSK3 inhibitor LiCl blocked the increase in ER-mediated transcription induced by the mutated form of p85. *, $P < 0.05$ *vs.* $\beta$E2 in the absence of LiCl in the p85Δ110-transfected group. E, N2a cells were cultured in medium containing 2% C/D FCS for 24 h and treated with vehicle (C) or the Akt inhibitor SH-6 (5 $\mu$M) for 15, 30, or 60 min. Phosphorylation of GSK3$\beta$ in serine 9 was analyzed using a phosphospecific antibody (pGSK3). Total GSK3$\beta$ was used as a control (GSK3). The Akt inhibitor SH-6 reduced the phosphorylation of GSK3$\beta$. F, N2a cells were transfected with pTA-ERE-SEAP and 24 h later treated with the Akt inhibitor SH-6 (1 or 5 $\mu$M) in the presence or absence of 10 mM LiCl (30-min pretreatment). Forty-five minutes later, $\beta$E2 (1 nM) was added to the medium for an additional 24 h. The graph shows the induction of reporter activity in each situation normalized to transfection efficiency. Data are presented as the mean ± SEM. SH-6 induced a dose-dependent increase in ER-mediated transcription. The GSK3 inhibitor LiCl blocked the effect of SH-6. *, $P < 0.05$ *vs.* the $\beta$E2- plus 5 $\mu$M SH-6-treated group.

### *ERα interacts with β-catenin in N2a cells*

In search of a possible mechanism of GSK3-mediated control of ER$\alpha$ activity, we studied the subcellular localization of both proteins. We performed immunofluorescence detection of both proteins using specific antibodies. ER$\alpha$ staining was mainly localized to the cell nucleus, whereas GSK3$\beta$ appeared to be located in the cytoplasm (Fig. 5A). We also analyzed the potential interaction of these two proteins in

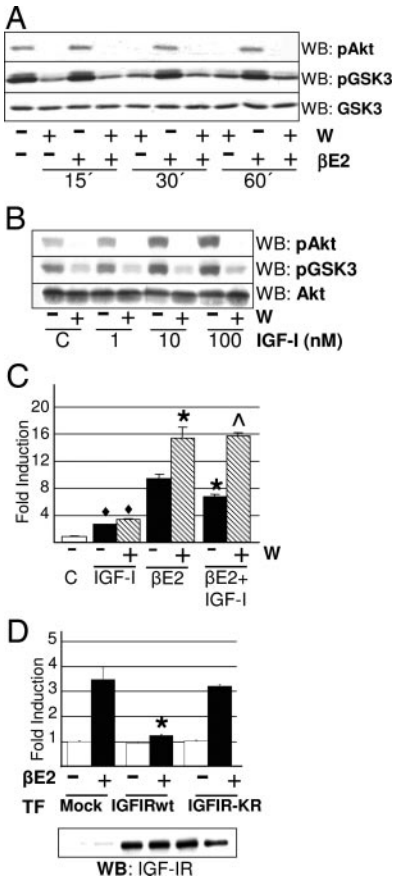


FIG. 4. IGF-I increases ER-dependent transcription in the absence of βE2 and decreases ER-dependent transcription, through PI3K, in the presence of βE2. A, To evaluate weather βE2 regulates Akt and GSK3 phosphorylation, N2a cells were cultured for 24 h in medium containing 2% C/D FCS, pretreated with wortmannin (W; 100 nM) or its vehicle for 30 min, and then treated for 0, 15, 30, or 60 min with 10 nM βE2. The activity of the Akt/GSK3 pathway was evaluated in total cell lysates by Western blotting using phosphospecific antibodies against serine 473 of Akt (pAkt; *upper gel*) and against serine 9 of GSK3β (pGSK3; *middle gel*). Total GSK3 (GSK3; *lower gel*) was used as a loading control. B, To assess whether endogenous IGF-IR in N2a cells regulates Akt and GSK3 phosphorylation, cells were cultured for 24 h in medium containing 2% C/D FCS, pretreated with wortmannin (W; 100 nM) or its vehicle for 45 min, then treated for 30 min with 0 [control (C)], 1, 10, or 100 nM IGF-I. The activity of the Akt/GSK3 pathway was evaluated in total cell lysates by Western blotting using phosphospecific antibodies against serine 473 of Akt (pAkt; *upper gel*) and against serine 9 of GSK3β (pGSK3; *middle gel*). Total Akt (*lower gel*) was used as a loading control. Treatment with IGF-I increased phosphorylation of Akt and GSK3, and this effect was blocked by the PI3K inhibitor wortmannin. C, To assess whether endogenous IGF-IR in N2a cells affects ER-mediated transcription, cells were transfected with pTA-ERE-SEAP and treated with vehicle (C), βE2 (1 nM), IGF-I (100 nM), or βE2 combined with IGF-I after 45 min of wortmannin (W) or vehicle pretreatment. Reporter activity was measured 24 h later and is presented as the mean ± SEM. Treatment with either IGF-I or βE2 alone resulted in a significant increase in reporter activity. In contrast, combined treatment with IGF-I and βE2 resulted in significantly lower reporter activity than treatment with βE2 alone. Wortmannin enhanced ER-mediated transcription in cells treated with βE2 or with both βE2 and IGF-I and prevented the inhibitory effect of IGF-I on βE2-induced, ER-mediated transcription. *, $P < 0.05$ *vs.* βE2 alone; ∧, $P < 0.05$ *vs.* βE2 plus IGF-I; ♦, $P < 0.05$ *vs.* the control group (C; the minimum significant difference test was applied in this case). D, N2a cells were cotransfected with pTA-ERE-SEAP and three different plasmids: an empty vector (Mock), the wt human IGF-IR (IGFIRwt), and a mutant, kinase-inactive IGF-IR (IGFIR-KR). Twenty-four hours after transfection, cells were treated with βE2 (1 nM) or its vehicle for an additional 24 h. SEAP activity was then quantified and normalized to transfection efficiency. The results shown are the mean fold induction in each situation ± SEM. Overexpression of the wt form of IGF-IR decreased βE2-induced, ER-mediated transcription. In contrast, ER-mediated transcription was unchanged by overexpression of the kinase-inactive form of IGF-IR. *, $P < 0.05$ *vs.* βE2 response in empty vector (Mock)-transfected group. The level of IGF-IR in each situation, as evaluated by Western blotting, is shown below.

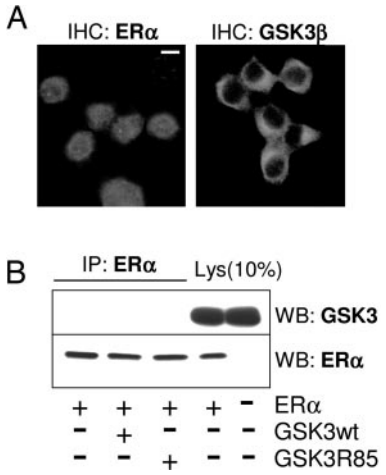


FIG. 5. ERα and GSK3β do not interact in N2a cells. A, N2a cells were cultured on gelatin-coated coverslips and in medium containing 2% C/D FCS for 24 h. Cultures were fixed and processed for the immunodetection of ERα and GSK3β. The signal from the Alexa Red 568-conjugated secondary antibody was analyzed in a confocal microscope. Representative photographs are shown. ERα immunoreactivity was mainly nuclear, whereas GSK3β immunoreactivity was located in the cytoplasm. *Scale bar*, 10 μm. B, N2a cells were transfected with overexpression plasmids for ERα and two forms of GSK3 (GSK3wt and GSK3R85) in the combinations described in the *lower part* of the figure. Cell lysates were immunoprecipitated with an antibody against ERα, and the immunoprecipitates were subjected to Western blotting using antibodies against GSK3β (GSK3; *upper gel*) and ERα (*lower gel*). Lys (10%), lanes loaded with 10% the immunoprecipitation input reveal the presence of endogenous GSK3β. Neither endogenous nor transfected forms of GSK3β were detected in the immunoprecipitates. The detection of large amounts of ERα in the immunoprecipitates confirms the efficiency of the immunoprecipitation.

N2a cells using immunoprecipitation. N2a cells were transfected with ERα- and GSK3 (wt and R85 mutated forms)-overexpressing plasmids. After 48 h, cultures were lysed and subjected to immunoprecipitation using antibodies against ERα and GSK3β. The immunocomplexes were resolved by Western blotting, and both proteins were detected using specific antibodies. As shown in Fig. 5B for immunoprecipitation using ERα antibody, we were not able to detect GSK3β in the immunocomplexes (Fig. 5B, *upper gel*), although the efficiency of the process was sufficient, as shown by the presence of large amounts of ERα (Fig. 5B, *lower gel*). We were also unable to detect ERα in the immunoprecipitates obtained with the GSK3β antibody (not shown).

β-Catenin is one of the most characterized mediators of GSK3 actions in the nucleus. For this reason, we hypothesized that this protein could be mediating GSK3 actions on ER activity. The stability of β-catenin is controlled by GSK3 in the context of a multimolecular complex that includes

adenomatous polyposis coli and axin (39). In N2a cells, PI3K and GSK3 activities control β-catenin levels in opposite ways. Western blotting analysis showed that blockade of PI3K with wortmannin lowered the β-catenin cellular content within 12 h (Fig. 6A, *upper panel*); in contrast, pharmacological inhibition of GSK3 (SB415286, 12 h) induced a robust increase in β-catenin expression (Fig. 6A, *lower panel*). The subcellular distribution of β-catenin was also affected by these treatments, as detected by immunofluorescence analysis of treated cells. GSK3 inhibition with SB415286 resulted in an increase in the nuclear localization of β-catenin, whereas this effect was not detectable in wortmannin-treated cells (Fig. 6B). In search of a possible mechanism of GSK3-mediated control of ER transcriptional activity, we assessed whether ERα and β-catenin interact in our cellular model. We detected colocalization of ERα and β-catenin in the cell nucleus of N2a cells. This colocalization was strongly increased by the GSK3 inhibitor SB415286 (Fig. 6B). In addition, an interaction between ERα and β-catenin in N2a cells was detected in immunoprecipitation experiments (Fig. 6C, *upper panel*). We also observed that this interaction could be modulated if βE2 was present in the immunoprecipitation buffer (Fig. 6C, *lower panel*). We then assessed whether treatment of N2a cells with βE2 or PI3K and GSK3 inhibitors regulated this interaction. With this aim, we performed immunoprecipitation in cells treated for various periods of time with βE2, wortmannin, and SB415286. The interaction between ERα and β-catenin was lowered to minimal levels after 45 min of βE2 treatment (Fig. 6D, *left panel*), returned to control values 1 h after addition of the hormone (Fig. 6D, *left panel*), and remained at basal levels 24 h after hormone treatment (Fig. 6D, *second panel*). The inhibition of PI3K with wortmannin also induced a decrease in the interaction between ERα and β-catenin that was detectable 1 and 10 h after the beginning of treatment (Fig. 6D, *third panel*). In contrast, inhibition of GSK3 with SB415286 induced an increase in the levels of β-catenin associated with ERα that were evident after 15 min of SB415286 treatment (Fig. 6D, *right panel*).

To test the role of β-catenin in the regulation that the PI3K/GSK3 pathway exerts on ER-mediated transcription, we used a mutant, nondegradable form of this protein in combination with reporter gene experiments. The mutation of serine 33 to a tyrosine (β-catenin S33Y) blocks GSK3 phosphorylation of this residue and thus prevents proteasome-dependent β-catenin degradation (23) and results in higher expression levels than the wild-type form (assessed by Western blotting; not shown). Probably as a consequence of this, the levels of ERα associated with the nondegradable mutant of β-catenin were also higher (Fig. 6E). Overexpression of the mutated form of β-catenin resulted in a similar induction of ER-mediated transcription as overexpression of the wt form. However, wortmannin did not enhance ER transcriptional activity in cells overexpressing mutant β-catenin, an effect that was evident in cells expressing wt β-catenin (Fig. 6F).

### *The PI3K/GSK3 pathway may regulate ERα stability in N2a cells*

In search of a possible mechanism for the control that PI3K and GSK3 exert on ER-mediated gene expression, we assessed whether these kinases regulate the expression of ERα protein in N2a cells. In this experiment we tested the effects of the three pharmacological inhibitors of GSK3 (LiCl, sodium valproate, and SB415286), and none of them nor overexpression of the wt or mutated form of this kinase affected the levels of endogenous ERα protein in N2a cells (Fig. 7A). We then analyzed the potential role of GSK3 in the regulation of ERα protein stability. To explore this possibility, N2a cells were plated, transfected with a plasmid constitutively expressing human ERα, and incubated in medium containing 2% C/D FCS for 24 h. The levels of ERα recombinant protein were evaluated by Western blotting at different times after pharmacological blockade of GSK3 or PI3K. Wortmannin induced a significant decrease in ERα expression, reaching 46 ± 1% of control levels after 6 h of treatment (Fig. 7B). Conversely, the GSK3 inhibitor SB415286 produced a significant increase in ERα protein expression, reaching 180 ± 18% of control levels 12 h after the initiation of treatment (Fig. 7B). The ability of these inhibitors to regulate ERα protein levels was also evident when they were coadministered with the ER antagonist ICI182780. This ER antagonist induced a rapid degradation of ERα (Fig. 7C, ■). Wortmannin increased the rate at which this degradation occurred, whereas GSK3 inhibition with SB415286 decreased it (Fig. 7C). To determine the effect of ERα protein stabilization on its transcriptional activity, we performed reporter gene experiments in the presence of MG132, a pharmacological inhibitor of the proteasome. Low concentrations (1 μM) of this compound selectively blocked ER-mediated transcription in N2a cells (Fig. 7D).

## Discussion

The results presented in this report show a new mechanism by which intracellular kinase signaling pathways may regulate gene expression. We used N2a cells as a model for estrogen actions in neural cells. As observed for many neurons in the rodent brain (3), these neuroblastoma cells express functional ERα. In our experiments, the activation of this subtype of ER is responsible for the majority of activation of the ER construct.

In contrast to what happens in other cell types (17), pharmacological inhibition of different members of the MAPK cascade, one of the main IGF-IR signaling pathways, has no significant effect on the βE2-induced activity of ERα in N2a cells. However, our results show evidence of the control that GSK3, one of the main effectors of the PI3K pathway, exerts on ER-mediated transcription in neuronal cells. Pharmacological inhibition of GSK3 decreased βE2-induced ERα transcriptional activity, suggesting that the activity of this kinase positively regulates the activity of the endogenous ER in N2a neuroblastoma cells. We used three different inhibitors, and all of them resulted in a decrease in ER-mediated transcription in a dose-dependent manner. LiCl and valproate did so in a concentration range consistent with their $EC_{50}$ values for GSK3 inhibition (1 mM for LiCl and 2 mM for valproate). For SB415286, the concentrations tested were higher than the inhibitory constant for GSK3 (31 nM). These concentrations were needed to block other GSK3-mediated effects on cortical neurons (40), N2a cells (41), and a liver-derived cell line

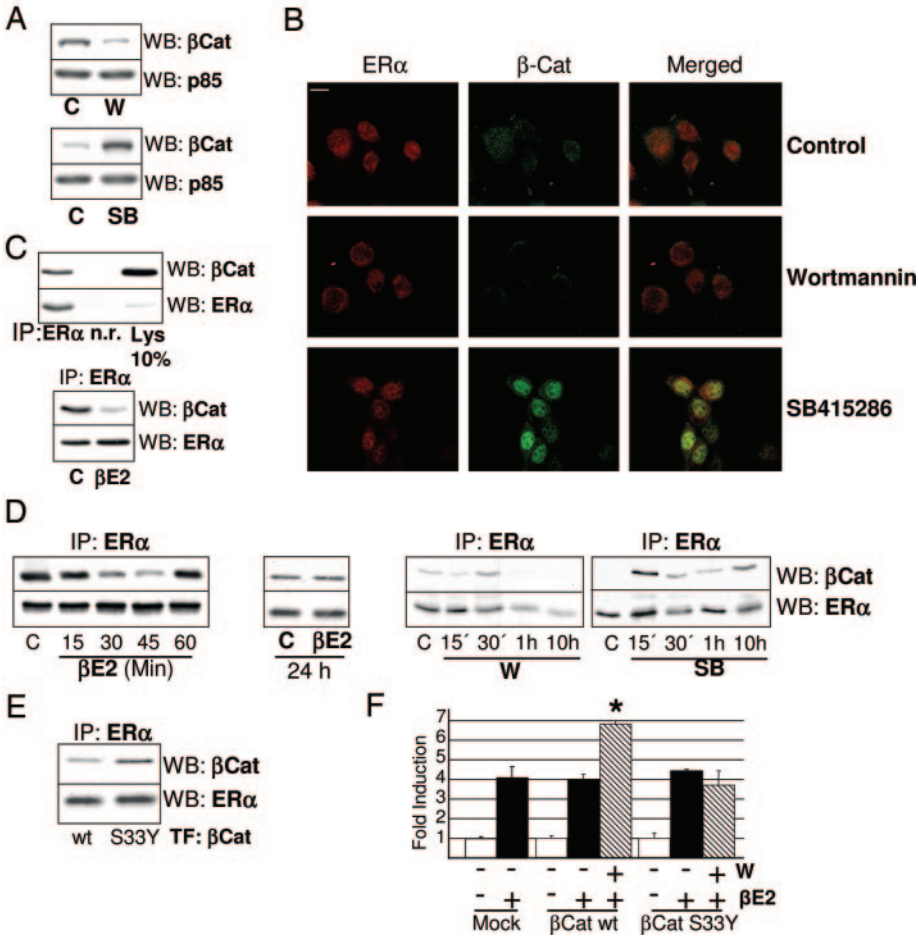


FIG. 6. β-Catenin interacts with ERα in N2a cells. A, N2a cells were cultured for 24 h in medium containing 2% C/D FCS, then treated with the PI3K inhibitor wortmannin (W; 100 nM) or the GSK3 inhibitor SB415286 (SB; 25 μM) for 12 h. The levels of β-catenin (βCat; *upper gels*) were evaluated by Western blotting in total lysates obtained from these cultures; p85 was used as a loading control (*lower gels*). A representative blot of three performed is shown. β-Catenin levels were decreased by the PI3K inhibitor wortmannin and were increased by the GSK3 inhibitor SB415286. B, Immunofluorescence analysis of β-catenin and ERα immunoreactivities in N2a cells. Cells were cultured in gelatin-coated coverslips in 2% C/D FCS for 24 h. Cultures were then treated with 100 nM wortmannin, 25 μM SB415286, or its vehicle (control) for an additional 12 h. Cultures were fixed, and ERα and β-catenin (β-cat) were detected using specific primary antibodies. The secondary antibodies were labeled with Alexa Red 568 for ERα and Alexa Green 488 for β-catenin. Representative photographs are shown. GSK3 inhibition with SB415286 resulted in an increase in nuclear localization of β-catenin and increased colocalization of β-catenin with ERα in the cell nucleus. This effect was not detectable in wortmannin-treated cells. *Scale bar*, 10 μm. C, Interaction between ERα and β-catenin in N2a cells in immunoprecipitation experiments. *Upper panel*, N2a cells were transfected with pHEGO and cultured for 24 h in 2% C/D FCS. Aliquots containing 300 μg protein were subjected to immunoprecipitation using an ERα-specific antibody or a nonrelated IgG (n.r.). The immunocomplexes were separated by electrophoresis and transferred to membranes where β-catenin was detected (βCat; *upper gel*). Ten percent of the immunoprecipitation input was subjected to Western blotting to verify the efficiency of the process (lane Lys 10%). In the same membranes, ERα was also immunodetected (*lower gel*). *Lower panel*, Cell lysates were subjected to immunoprecipitation with an ERα antibody in the presence of 100 nM βE2 or its vehicle in the immunoprecipitation buffer. The immunocomplexes were resolved and probed for the presence of β-catenin (βCat; *upper gel*) and ERα (*lower gel*). Representative gels are shown. The presence of βE2 in the immunoprecipitation buffer decreased the amount of immunoprecipitated β-catenin. D, Effect of βE2 treatment on the interaction of ERα and β-catenin. N2a cells were cultured, transfected with pHEGO, and treated with βE2, wortmannin (W; 100 nM), or SB415286 (SB; 25 μM) for the indicated periods of time before lysis. Cell lysates were subjected to immunoprecipitation and Western blotting as described in C. Representative gels from three runs performed are shown. *Left panel*, A transient decrease in the amount of immunoprecipitated β-catenin was detected 45 min after the addition of βE2 to the cultures. Immunoprecipitated β-catenin levels returned to control values 1 h after addition of hormone (*left panel*) and remained at control levels 24 h after treatment (*second panel*). *Third panel*, Inhibition of PI3K with wortmannin induced a decrease in the interaction between ERα and β-catenin that was detectable 1 and 10 h after the beginning of treatment. *Right panel*, A rapid increase in the level of β-catenin associated with ERα was detected after inhibition of GSK3 with SB415286. E, ERα interaction with mutant β-catenin. N2a cells were cotransfected with the expression plasmid for ERα and the expression plasmids for wt β-catenin (wt) or a mutated, nondegradable form (S33Y). Cell lysates were subjected to immunoprecipitation and Western blotting as described in C. Representative gels from two runs are shown. Interaction between ERα and both forms of β-catenin was observed. Indeed, the mutated form showed a stronger interaction with ERα than the wt form. F, Cells were cotransfected with pTA-ERE-SEAP and the expression plasmids for empty vector (Mock), wt β-catenin (βCat wt), or the mutated, nondegradable form (βCat S33Y). Cells were then pretreated with 100 nM wortmannin (W) or its vehicle before the addition of 1 nM βE2 for 24 h. SEAP activity was quantified and normalized to transfection efficiency. The results are the mean fold induction in each situation ± SEM. The PI3K inhibitor wortmannin enhanced ER-mediated transcription in cells overexpressing wt β-catenin. In contrast, wortmannin did not affect ER-mediated transcription in cells overexpressing the nondegradable form of β-catenin. *, $P < 0.05$ *vs.* βE2 in the βCat wt group.

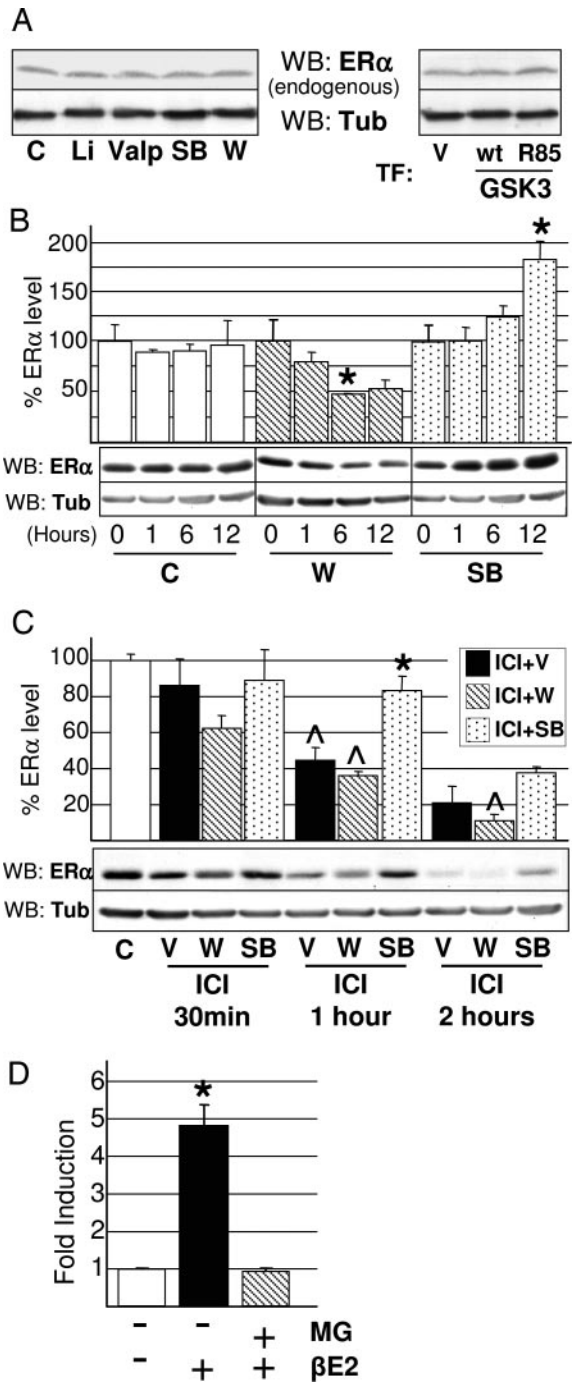


FIG. 7. The PI3K/GSK3 pathway regulates ERα protein stability. A, N2a cells were treated for 24 h with 20 mM LiCl (Li), 3 mM sodium valproate (Valp), 25 μM SB415286 (SB), and 100 nM wortmannin (W) or transfected (TF) for 48 h with wt GSK3, mutant GSK3 (GSK3 R85), or an empty vector (V). None of these treatments affected the level of endogenous ERα in N2a cells, evaluated by Western blotting with a specific antibody against mouse ERα (MC20). βIII-Tubulin (Tub) was used as a loading control. B, N2a cells were transfected with expression plasmids for human ERα (pHEGO), cultured for 24 h in medium containing 2% C/D FCS, and treated with vehicle (C), wortmannin (W; 100 nM), or SB415286 (SB; 25 μM) for 0, 1, 6, or 12 h. Levels of recombinant ERα were evaluated by Western blotting using an antibody that recognizes human ERα. βIII-Tubulin (Tub) was used as a loading control. Data are the mean ± SEM. A significant decrease in ERα protein expression was detected 6 h after treatment with the PI3K inhibitor wortmannin. In contrast, treatment with the GSK3 inhibitor resulted in a significant increase in ERα protein expression 12 h after the addition of SB415286 to the cultures. *, $P < 0.05$ *vs.* the control group in each treatment. C, N2a cells were transfected as described in B and preincubated for 1 h with 100 nM wortmannin (W), 25 μM SB415286 (SB), or vehicle (V). Cells were then treated with the ER antagonist ICI182780 (ICI; 200 nM) for 30 min, 1 h, or 2 h. ERα levels were evaluated as described in B and normalized to the level of ERα in each group at the beginning of ICI treatment (C). Data are the mean ± SEM. The ER antagonist induced a rapid degradation of ERα that was accelerated by wortmannin and delayed by the GSK3 inhibitor SB415286. *, $P < 0.05$ *vs.* the control (V) at each time point; ^, $P < 0.05$ *vs.* SB415286-treated cells (SB) at each time point. D, N2a cells were transfected with pTA-ERE-SEAP. Thirty minutes before βE2 addition, cultures were treated with the proteasome inhibitor MG132 (MG; 1 μM) or its vehicle. Reporter activity was evaluated 24 h later. Data, after normalization, are the mean ± SEM. The proteasome inhibitor MG132 blocked the induction of ER-mediated transcription by βE2. *, $P < 0.05$ *vs.* the control (vehicles).

(32). The different concentrations of SB415286 needed to inhibit GSK3 activity *in vitro* and to block GSK3 effects in cells may reflect the partial cell permeability of this compound. In particular, it is possible that SB415286 may have restricted access to the nuclear compartment, where it may exert its effects on transcription. In addition, it should be noted that the inhibitors used in this study are not fully specific for GSK3. At the concentration used in our experiments, important targets, such as inositol monophosphatase for LiCl (42) and γ-aminobutyric acid (43) or histone deacetylases (44) for valproate could be partially responsible for the effects on ERα-mediated transcription in N2a cells. We cannot exclude that these additional actions of valproate and LiCl may be involved in the different effects of the three inhibitors on ERα-mediated transcription in HEK293 cells and on RAR-mediated transcription in N2a cells. Nevertheless, the only known shared target of LiCl, valproate, and SB415286 is GSK3, suggesting that this kinase is mediating the analogous effects of these compounds on ERα-mediated transcription in N2a cells. A role for GSK3 in the regulation of ER-mediated transcription is also suggested by the increased transcriptional activity observed after overexpression of GSK3 in N2a cells. The significant increase in ERα activity (40%) induced by GSK3 transfection was not insubstantial considering the high endogenous basal levels of expression of GSK3 in N2a cells. Furthermore, the enhancement of ERα-mediated transcription was not observed when a mutated, kinase-inactive form of GSK3 (GSK3βR85) (22) was transfected, suggesting the possible involvement of GSK3 in the regulation of ERα activity.

Surprisingly, the transcriptional activity of RAR in N2a cells was not inhibited by any of the three GSK3 inhibitors used in our study. Moreover, treatment with LiCl and SB415286 stimulated the response of the RAR-sensitive construct. This suggests that the effect of GSK3 is specific for ERα and different from its effect on the activities of other members of the superfamily of nuclear receptors to which both ER and RAR belong. Interestingly, this same phenomenon has been described for Akt in the control of these two nuclear receptors (16). In addition, the control exerted by GSK3 on ER-mediated transcription is also cell type dependent, because the inhibition of ERα activity by GSK3 blockade was not present in HEK293 cells. Indeed, two of the GSK3 inhibitors used,

LiCl and valproate, stimulate ERα-mediated transcription in HEK293 cells. Interestingly, IGF-I has a positive stimulatory effect on ERα-mediated transcription in this cell line (45).This is in agreement with the tissue- and cell-specific effects described for transcriptional effects of estrogen and could be related to the differential recruitment of transcriptional coregulators in response to ER activation (46). A similar cell type-specific effect of GSK3 in the control of nuclear receptor-mediated transcription has been described previously for androgen receptors (47, 48).

The Akt inhibitor SH-6 (38) and the PI3K inhibitor wortmannin induced a strong decrease in serine 9 phosphorylation of GSK3β, suggesting that there is a functional link among GSK3, Akt, and PI3K in N2a cells (36). Pharmacological or genetic inhibition of PI3K strengthened the response of the estrogen-sensitive reporter construct. This suggests that PI3K is a negative modulator of ER activity in N2a cells, in contrast to what has been described in other cell types (16, 49). Inhibition of GSK3 blocked the stimulatory effects of Akt and PI3K inhibition on the transcriptional activity of ERα in N2a cells, indicating that changes in PI3K/Akt activities are translated to changes in ER transcription by means of GSK3. Our findings suggest that βE2 is not interfering with this mechanism, at least not by direct regulation of the activity of the PI3K/Akt/GSK3 pathway, because Akt and GSK3 phosphorylation is not affected by βE2 treatment in N2a cells. In contrast, the regulation of nuclear ERα activity by GSK3 could be elicited by extracellular treatment with IGF-I or overexpression of IGF-IR in N2a cells. IGF-IR activation by ligand binding in the absence of E2 elicited a rapid increase in Akt (serine 473) phosphorylation, a subtle increase in GSK3β (serine 9) phosphorylation, and a delayed increase in the activity of ERα. The increase in ERα activity by IGF-I in N2a cells is similar in magnitude to that elicited by IGF-I in other neuroblastoma cell lines (7). In addition, as has been described for the effect of insulin in other neuroblastoma cells (50), the increase in ERα activity induced by IGF-I in the absence of βE2 is independent of PI3K activity. In contrast, when IGF-I was applied simultaneously with βE2, it reduced the response elicited by estrogen alone by approximately 30%. This inhibitory effect of IGF-I was mediated by the PI3K pathway, as suggested by its blockade by wortmannin treatment. Thus, in N2a cells, as in other cell types (7, 50), IGF-I increases ERα transcriptional activity in the absence of βE2. However, the effect of IGF-I is different in the presence of βE2. In this case, IGF-I decreases ERα transcriptional activity. Therefore, our results suggest that the regulation of ERα activity by IGF-I is different depending on whether the ERα ligand is present and that this differential regulation of ERα activity is possible in the same cell type.

The mechanisms involved in the regulation of transcription factors by GSK3 are still unclear, but normally involve direct phosphorylation (19). Because GSK3 and ERα localize to different cellular compartments in N2a cells, and we have been unable to detect a direct interaction between these two proteins (51), we hypothesized that GSK3 controls ERα-mediated transcription through an alternative mechanism. Our findings suggest that the cross-talk between GSK3 and ER is mediated by β-catenin. The level of expression and the nuclear localization of this protein in N2a cells are controlled in opposite ways by PI3K and GSK3 activities. In addition, this protein interacts with ERα in a regulated manner in N2a cells. Our results indicate that high levels of β-catenin (total and associated with ERα) are negatively correlated with ER transcriptional activity in N2a cells. Although the nondegradable β-catenin mutant (β-catenin S33Y) had no effect on ERα transcription under basal conditions, its overexpression completely blocked wortmannin-induced stimulation of ERE-dependent activity. It is interesting to note that ERα showed greater interaction with the mutated form of β-catenin than with the wt form. It is tempting to speculate that the increased binding of the mutated form of β-catenin to ERα may be involved in its inhibitory effect on wortmannin-induced stimulation of ER activity. The role of the interaction with β-catenin on the transcriptional regulation by ERα needs additional investigation to be clarified, but our results suggest that β-catenin is part of the signaling pathway by which changes in PI3K/GSK3 activity are translated into modifications of ERα transcriptional activity in N2a cells. The regulation of this interaction by βE2 raises the important question of the role of transcription in the stability of the ERα/β-catenin complex. The rapid down-regulation of the interaction between ERα and β-catenin in N2a cells after βE2 treatment, which is evident at 30–45 min, and the rapid recovery to control levels, which is observed 1 h after hormone treatment, suggest that the decrease in stability of the ERα/β-catenin complex precedes the first round of ER-mediated transcription (52). Therefore, transcriptional activity is probably not involved in destabilization of the ERα/β-catenin complex. An alternative possibility is that the decreased interaction between ERα and β-catenin may represent an early event in the regulation of ER-mediated transcription.

The convergence between β-catenin and ERα has been demonstrated in other cell systems, but with different functional outcomes (53). In the MCF-7 cell line, the overexpression of the β-catenin S33Y mutant enhances ERE-mediated transcription (53). This suggests a cell type specificity of ERα/β-catenin interaction that, as we shown here and previously reported (54), is negatively regulated by βE2 and has an inhibitory role in ER-mediated transcription in neural cells and tissue. In addition, in MCF-7 cells, the ERα/β-catenin interaction has been shown to regulate the transcriptional activities of both proteins (53). This reciprocal control raises the possibility of a regulatory role of ER in β-catenin-mediated gene expression in neural cells.

Because degradation has emerged as an important regulatory mechanism governing the transcriptional activity of ERα, we decided to specifically explore the posttranscriptional mechanism governing ERα stability in N2a cells. The experiments using plasmids constitutively expressing ERα allowed us to specifically study this aspect of ERα protein expression. Recent evidence shows that proteolysis is essential for ERα-mediated transactivation, and that proteasome-dependent turnover of ERα is an integral characteristic of ER activity (55). MG132, a proteasome inhibitor that interferes with ERα degradation (55), completely blocked ERE-mediated gene expression in N2a cells. This suggests that there is a functional link between ER degradation and activity in this cell line also. Interestingly, the PI3K/GSK3 pathway seems to affect the stability of unliganded, and antagonist-bound,

recombinant ERα in a manner compatible with this idea. The GSK3 inhibitor SB415286, which blocked ER-mediated transcription, induced a substantial increase in recombinant ERα protein levels, probably through the blockade of basal and ICI182780-induced degradation of ERα. In contrast, activation of GSK3 by wortmannin accelerated the degradation rate, producing a decrease in ERα protein levels. These results suggest that the mechanism by which the PI3K/GSK3 pathway regulates ERα-mediated transcription includes the control of ERα proteolysis and turnover. This does not necessarily imply that the PI3K/GSK3 pathway affects total ERα levels in the cells. Indeed, our findings indicate that the expression levels of endogenous ERα are independent of PI3K and GSK3. This suggests that other cellular processes regulating ERα synthesis and degradation may be differentially affected, directly or indirectly, by the PI3K/GSK3 pathway to maintain stable levels of ERα expression. Because the levels of ERα and β-catenin are regulated in the same direction by PI3K and GSK3 activities in N2a cells, it is tempting to speculate that the interaction between these two proteins may be crucial for regulating ERα proteolysis. The results presented in this report are consistent with a protective role of β-catenin in ERα proteolysis that may be responsible for the inhibition of ERα-mediated transcription. However, additional studies are needed to clarify this point.

In summary, our results suggest that ERα transcriptional activity is regulated by intracellular kinases in N2a cells. PI3K and Akt, through changes in GSK3 kinase activity, may modulate ERE-dependent gene expression. This interaction seems to be mediated by β-catenin, which interacts in a regulated manner with ERα. In addition, PI3K and GSK3 activities regulate ERα protein stability and antagonist-induced degradation. Therefore, estrogen may be intimately associated with kinase-signaling mechanisms in neuronal cells. Through its interaction with GSK3, estrogen could be regulating cytoskeleton dynamics, neuronal plasticity, and neurodegenerative processes, all of which are targets of GSK3 activity in neuronal cells (19). In addition, our results represent a new mechanism by which extracellular factors that regulate PI3K, Akt, and GSK3 activities could be affecting the transcriptional activity of ERs. Thus, our findings suggest that IGF-I decreases ERα transcriptional activity in N2a cells via the PI3K/Akt/GSK3 signaling pathway in the presence of βE2. In contrast, in full agreement with what has been previously reported in other neuroblastoma cells (7, 50), IGF-I increases the activity of the unliganded ERα in N2a cells by a mechanism that is not mediated by this signaling pathway. Therefore, the regulation of ER transcriptional activity by IGF-I may differ depending on the concentration of βE2. This dual mechanism may have many implications for the physiological and pathological actions of IGF-I and βE2 in the brain and other organs. However, additional studies in other model systems are necessary to determine whether this mechanism is applicable to other cell types and whether it operates in the nervous system.

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P.M. and L.M.G.S. have nothing to declare.

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