Glycogen Synthase Kinase-3 Interacts with and Phosphorylates Estrogen Receptor α and Is Involved in the Regulation of Receptor Activity

Senad Medunjanin, Alexander Hermani, Barbara De Servi, Jean Grisouard, Gabriele Rincke, and Doris Mayer

From the Hormones and Signal Transduction Group, German Cancer Research Center, 69120 Heidelberg, Germany

Like other steroid hormone receptors, estrogen receptor-α (ERα) is a substrate for protein kinases, and phosphorylation has profound effects on the function and activity of this receptor. A number of different kinases have been implicated in ERα regulation. In this report we show by mutational analysis and in vitro kinase assays that ERα is a substrate for glycogen synthase kinase-3 (GSK-3) and is phosphorylated on two sites, the Ser-102, -104, and -106 motif and Ser-118, both located in the N-terminal transcription activation function (AF-1) domain. GSK-3 forms a complex with ERα in vivo as demonstrated by co-immunoprecipitation from cell lysates. The GSK-3 inhibitor lithium chloride was used to determine the role of GSK-3 in phosphorylation of Ser-102, -104, and -106 and Ser-118 in vivo and to explore the role of these serines in the regulation of ERα function. Treatment of cells with lithium chloride resulted in dephosphorylation of Ser-104 and -106 and Ser-118, which suggests that these serine residues act as targets for GSK-3 in vivo. Our results further suggest that ERα phosphorylation by GSK-3 stabilizes ERα under resting conditions and modulates ERα transcriptional activity upon ligand binding. Inhibition and constitutive activation of GSK-3, both, resulted in inhibition of ERα transcriptional activity, indicating a function of active as well as inactive GSK-3 in ERα regulation. These findings uncover a novel mechanism for the regulation of ERα-mediated estrogen signaling controlled by a dual action of GSK-3.

Estrogen receptor-α (ERα) is a member of the nuclear receptor superfamily of transcription factors, which are activated by small lipid-soluble molecules including steroid hormones (1). Nuclear receptors are characterized by a conserved structural and functional organization and play essential roles in development, differentiation, and metabolism by controlling the expression of specific networks of genes (2, 3). Based on sequence homology and other approaches, the estrogen receptor protein can be divided into six functionally and physically independent domains, nominated A to F (1, 2), that encode sequences required for DNA binding (region C), nuclear localization (region D), and ligand binding (region E). Ligand binding results in conformational change of ERα, formation of homodimers, translocation into the nucleus, and subsequent binding to estrogen response elements (EREs) located within regulatory regions of target genes (4, 5). The ERα has two well characterized transcriptional activation functions: AF-1, which is located in the N-terminal A/B region and may be activated in a ligand-independent manner, and AF-2, which is located in region E and whose activity is ligand-dependent. AF-1 and AF-2 can activate transcription independently and synergistically act in a promoter- and cell type-specific manner (4, 5).

The ERα, like other members of the steroid hormone receptor superfamily, is phosphorylated on multiple serine residues (6, 7). Serine phosphorylation is essential for transcriptional activation in response to estradiol binding and occurs on Ser-167 and Ser-118, the latter being the major phosphorylation site required for full activation of ERα and to a lesser extent on Ser-104 and Ser-106, all located in the AF-1 domain of the receptor (7). TFIIH kinase Cdk7 (8) and mitogen-activated protein kinase (9) have been reported to phosphorylate Ser-118, and cyclin A-Cdk2 was associated with Ser-104 and -106 phosphorylation (10). Recent work suggested a role of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in estrogen-dependent activation and nuclear translocation of ERα (11–13).

A protein kinase that has been observed to interact with ERα in rat hippocampus (14) and, therefore, may be related to estrogen signaling is glycogen synthase kinase-3 (GSK-3). GSK-3 is a proline-directed serine/threonine kinase (15) that is widely expressed in mammalian tissues. Unlike most kinases, GSK-3 is catalytically active in resting cells (16, 17). GSK-3 is a crucial regulator of embryonic development as well as of numerous cellular functions in adult tissues such as glycogen metabolism, lipid synthesis, cytoskeletal arrangement, apoptosis, gene expression, and proliferative response, and is implicated in human diseases including cancer, Alzheimer disease, and diabetes (16). The two mammalian GSK-3 isoforms (GSK-3α and GSK-3β) are stringently controlled in response to growth factor or hormonal stimulation by phosphorylation, protein complex formation, and subcellular distribution (16–18). Both GSK-3 isoforms have a similar substrate specificity; however, the disruption of the GSK-3β gene in mice results in embryonic lethality, indicating that GSK-3α cannot completely compensate the loss of GSK-3β (19). GSK-3 activity is significantly reduced by phosphorylation on Ser-21 in GSK-3α and Ser-9 in GSK-3β. Studies in different cells suggest that the protein kinases p70S6 kinase, p90Rsk, Akt/protein kinase B, protein kinase A, and certain protein kinase C isoforms phosphorylate Ser-9 in GSK-3β and Ser-21 in GSK-3α (17, 20, 21).

We show here that ERα is a substrate of GSK-3, being phosphorylated at two different sites in the AF-1 domain in response to estradiol (E2). This suggests an important function of GSK-3 in the regulation of ERα transcriptional activity.
Phosphorylation of ERα by GSK-3

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—MELN and COS-1 cells were grown in phenol red-free Dulbecco’s modified Eagle’s medium (4.5 g/liter glucose) (Invitrogen) containing 10% dextran-coated charcoal-treated serum (22) for 48 h before treatment with E2 or inhibitors. Transfections of MELN cells were performed by electroporation using Gene Pulser II (Bio-Rad) at 975 microfarads, 250 V. COS-1 cells were transfected by the DEAE-dextran method (23). Briefly, COS-1 cells were grown to ~60% confluence. Plasmid DNA (3 μg) and DEAE-dextran (0.2 mg/ml in phosphate-buffered saline) were added to the COS-1 cells for 30 min at 37 °C followed by the addition of Dulbecco’s modified Eagle’s medium containing 100 μM chloroquine and an additional incubation for 3 h. Then the solution was aspirated, and 10% Me2SO in phosphate-buffered saline was added for 2 min following by rinsing and incubation at normal growing conditions. MDA-MB231 cells were grown in phenol red-free RPMI 1640 (PAA, Colbe, Germany) and transfected using jetPEI (Polyplus-Transfection, Illkirch, France) according to the manufacturer’s instructions. Transfection efficiency of all cell lines was proven by a green fluorescent protein control vector.

Antibodies—Monoclonal antibody to ERα (NCL-L-6F11) was from Novocastra (Newcastle, UK), rabbit polyclonal antibodies to ERα (HC-20) and to cyclin D1 were from Santa Cruz Biotechnology. Phosphospecific anti-α-Ser3,37,41-β-catenin (#9561), anti-β-catenin (#9562), anti-Thr(286)-cyclin D1 (#9291), anti-pSer-118-ERα (#2511), and anti-GSK-3α/β (#44–610) was from BIO-RAD. rabbit IgGs were from Dianova (Hamburg, Germany). Non-immune IgGs were from Upstate Biotechnology (Charlottesville, VA).

Microscopy—MELN cells were grown on poly-d-lysine-coated glass coverslips to 50% confluence, kept in medium with dextran-coated charcoal-treated serum for 48 h, and treated with E2 (100 nM) for 20 min. Cells were fixed in methanol for 5 min and acetone for 1 min at ~20 °C and then processed for double immunofluorescence staining of GSK-3 and ERα. ERα was detected using a rabbit antibody directed against the C terminus of human ERα, and GSK-3-α/β was detected with a mouse antibody. All antibody incubations were performed in phosphate-buffered saline for 1 h at room temperature. Alexa-green conjugated anti-mouse IgG was from Molecular Probes (Eugene, OR), Cy3-conjugated anti-rabbit IgG and horseradish peroxidase-labeled anti-mouse and anti-rabbit IgGs were from Dianova (Hamburg, Germany). Non-immune IgGs were from Upstate Biotechnology (Charlottesville, VA).

Immunoprecipitation and SDS-PAGE—Cells lysates (50 μM Hesper at pH 7.6, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1.5% Triton X-100, 1 mM sodium orthovanadate, 1 mM NaF, complete protease inhibitor mixture (Roche Applied Science) were cleared by centrifugation (10,000 × g, 10 min). Lysates containing equal amounts of proteins were precleared with IgG bound to protein A-agarose beads (Sigma) for 2 h at 4 °C and immunoprecipitated with the specific primary antibody and protein A-agarose overnight with gentle agitation. The precipitates were subjected to SDS-PAGE and immunoblotting using phosphospecific primary antibodies and horseradish peroxidase-labeled secondary antibodies. Immunoreactive bands were detected by the ECL-plus reagent (Amersham Biosciences). After stripping, the membranes (Immobilon P, Millipore, Eschwege, Germany) were probed with antibodies to the respective protein.

Cellular Fractionation—Cells were homogenized in Nuclear Buffer I (10 mM Hepes at pH 7.8, 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.6% Triton X-100) containing the complete protease inhibitors mixture (Roche Applied Science) using a glass homogenizer. After centrifugation (1000 × g at 4 °C), the supernatant (cytoplasmic fraction) was collected. The pellet was washed twice in Nuclear Buffer II (50 mM Hepes at pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol), incubated on ice for 30 min, and centrifuged at 15,000 × g for 10 min. The supernatant (nuclear proteins) and the cytoplasmic fraction were subjected to SDS-PAGE and immunoblotting.

Plasmids—The GSK-3β cDNA (a gift from Dr. P. Angel) was subcloned into pcDNA3 (Invitrogen). The GSK-3β9A mutant was generated by PCR using the wild type construct as a template (forward primer, 5’-GCTAGCATGTCAAGGGCGGCCCAGACACCGCTTT-3’; reverse primer, 5’-TATAGGCTGGAGGGTGAAGCTGATG-3’), and the PCR reaction product was cloned into pcDNA3. The full-length cDNA of human ERα was cloned into pcDNA3 2×FLAG (pcDNA3 ERα2×FLAG). Site-directed mutagenesis of the ERα (serine to alanine) was performed using the QuickChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA). Mutations were verified by DNA sequence analysis using the ABI PRISM® BigDye™ Terminator Ready reaction cycle sequencing kit and ABI PRISM® 377 DNA Sequencer (Applied Biosystems, Foster City, CA).

Expression of Recombinant Proteins—cDNA fragments coding for amino acids 76–176 of the B domain of wild type and mutant ERα were amplified by PCR from the respective pcDNA3 ERα2×FLAG constructs. The PCR fragments (GST-ERα76–176) wild type and GST-ERα76–176 mutants) were inserted into the pGEX-4T vector (Amersham Biosciences). Overnight cultures of Escherichia coli BL21 transformed with the GST constructs or the GST control plasmid were diluted 100-fold, cultured for 5–6 h, and then induced with 0.1 mM isopropyl-β-thiogalactopyranoside for 3 h. GST-ERα fusion proteins were purified using glutathione-Sepharose as described by the manufacturer (Amersham Biosciences).

In Vitro Phosphorylation Assay—Recombinant human ERα (Panvera, Madison, WI) or purified GST-ERα76–176 fusion proteins (wild type and mutants) were incubated with rabbit GST-3β3 (New England Biolabs) for 10 min at 30 °C in a total volume of 30 μl of protein kinase assay buffer (200 μM ATP, 25 mM Tris-HCl at pH 7.4, 5 mM MgCl2, 0.5 mM EGTA, 1 mM dithiothreitol, 10 mM β-mercaptoethanol) containing 1 μCi of γ-32P]ATP (6000 Ci/mmol). Phosphoprotein products were detected by PAGE (12% gel), Coomassie Blue staining, and autoradiography.

Luciferase Assays—MELN cells were washed with phosphate-buffered saline (Mg2+ - and Ca2+ -free) and lysed in 150 μl/well luciferase cell culture lysis reagent (Promega, Mannheim, Germany). Luciferase assays were performed using the firefly luciferase assay system from Promega according to the manufacturer’s instructions and a luminometer (Biolumat LB6505, Berthold, Bad Wildbad, Germany).

RESULTS

GSK-3 Is Involved in ERα Activation—Based on findings that estra-diol treatment of cells resulted in activation of Akt/protein kinase B in...
Phosphorylation of ERα by GSK-3

FIGURE 1. GSK-3 modulates transcriptional activation of ERα. A, MELN cells were incubated for 48 h with E2 (10^{-8} M), ICI 182,780 (I), LiCl (3 × 10^{-2} M) and combinations thereof. ERα-dependent gene expression was quantified by measuring luciferase activity. Data are expressed as -fold luciferase activity measured in untreated cells. Error bars represent S.D. of two experiments (four measurements). B, MELN cells were stimulated with E2 (10 min), lysates were analyzed by immunoblotting (IB) with anti-phospho-GSK-3α/β (pGSK-3α/β) and anti-GSK-3α/β. C, cells were treated with E2 (10^{-7} M), and lysates were immunoblotted using anti-pGSK-3α/β and anti-GSK-3α/β.

estrogen-dependent MCF-7 cells (12), we hypothesized that GSK-3, a substrate and down-stream effector of Akt/protein kinase B, is involved in the regulation of ERα function. We investigated the role of GSK-3 in estradiol-dependent transcriptional activation of ERα. MELN cells (derived from MCF-7 breast cancer cells by stable transfection with a luciferase reporter gene under the control of an estrogen response element using the β-globin promoter (24)) were treated with E2 and with the GSK-3 inhibitor LiCl (25), and luciferase activity was measured. E2 treatment resulted in a ~4-fold induction of luciferase activity. This effect was inhibited by the specific ER-antagonist ICI 182,780 studied for control and by LiCl (Fig. 1A). The reduction of estrogen-dependent ERα transcriptional activity by LiCl indicated a function of active (unphosphorylated) GSK-3 in ERα activation. E2 treatment of cells resulted, however, in GSK-3 phosphorylation (inactivation) in a dose (Fig. 1B)- and time-dependent manner (Fig. 1C) that agrees with findings on inactivation of GSK-3 by E2 in rat hippocampus (14). These observations on GSK-3 inactivation by E2 treatment and the decrease in E2-induced ERα transcriptional activation by the GSK-3 inhibitor were, at first glance, surprising and contradictory. However, the results implied the possibility of multiple functions of GSK-3 regarding ligand-dependent ERα activation.

GSK-3 Associates with Human ERα—GSK-3 has been reported as a docking kinase (26), able to form complexes with substrates in different pathways. To clarify if ERα is a physiological substrate for GSK-3, we investigated whether ERα and GSK-3 can physically associate in vivo. The interaction between GSK-3 and ERα was assessed by immunoprecipitation of endogenous GSK-3α/β or ERα and analyzing the immune complexes for the presence of ERα or GSK-3β, respectively. We observed an association of GSK-3α and ERα in untreated cells that increased after E2 treatment, showing a maximum at 20 min (Figs. 2, A and B). This association was confirmed by immunofluorescence and co-localization assays, two different antibody sets yielding similar results (Fig. 2C). Co-localization of ERα and GSK-3 was observed mainly in the cytoplasm of untreated cells and in the nuclei in E2-treated cells. GSK-3β that co-precipitated with ERα (Fig. 2B) was not phosphorylated at Ser-9, meaning that ERα interacted with the active form of GSK-3β. We, therefore, were interested in the implications of GSK-3 phosphorylation/inactivation observed after E2 treatment on ERα activity. Previous reports suggest that only a small and specific pool of GSK-3 present in the cell is phosphorylated after a specific stimulus depending on the subcellular distribution of GSK-3 (16). To localize the cellular site of GSK-3 phosphorylation under E2 influence, we fractionated cells at various time periods after E2 treatment. E2 stimulation resulted in a rapid phosphorylation of cytoplasmic GSK-3, whereas GSK-3 located in the nucleus remained unphosphorylated (Fig. 2D). At 20 min of E2 treatment, when maximum interaction of GSK-3β and ERα was observed (Fig. 2B), ERα had translocated into the nucleus where it interacted with nuclear GSK-3β (Fig. 2C), which was in the unphosphorylated (active) state. It is concluded that ERα forms complexes with active GSK-3β in both unstimulated and E2-stimulated cells.

The ERα domain mediating the interaction between GSK-3 and ERα was mapped by generating truncated mutants lacking the A, AB, and F domains of the receptor (Fig. 3A). After co-expression of the mutants with GSK-3β in ER-negative COS-1 cells, all ERα mutants were co-immunoprecipitated with GSK-3β except that lacking the AB domain, revealing the B domain of ERα as the domain interacting with GSK-3 (Fig. 3B).

GSK-3 Phosphorylates ERα at Two Motifs in Vitro—The interaction of active endogenous GSK-3 with ERα in unstimulated MELN cells and of ERα and GSK-3β expressed in COS-1 cells suggested ERα to represent a substrate for GSK-3 acting as a docking kinase. This was confirmed by a radioactive in vitro kinase assay using recombinant human ERα and rabbit GSK-3β in which a strong phosphorylation of ERα was obtained (Fig. 4A).

Next, we identified the serine residues of ERα that are phosphorylated by GSK-3. It has been reported that GSK-3 recognizes two different substrate motifs characterized by either primed or non-primed phosphorylation sites (17). Primed substrates of GSK-3 are phosphorylated by another kinase at a Ser/Thr site that is located four amino acids C-terminal to the target site, which primes the substrate for phosphorylation by GSK-3. This primed motif (S/T)XXX(pS/pT; p5 and pT are phosphorylated Ser and Thr) is found in proline-rich regions of substrates such as glycogen synthase (27), CREB (28), and β-catenin (29).
Although the majority of GSK-3 substrates seem to be primed, GSK-3 also phosphorylates non-primed sites at (Ser/Thr)-Pro motifs. Examples of non-primed substrates are cyclin D1 (30), Tau (31) (Fig. 4C), axin, and the adenomatous polyposis coli (APC) gene product.

Amino acid sequence comparison of the ERα B domain and known GSK-3 substrates (Figs. 4, B and C) revealed two putative GSK-3 phosphorylation sites in the ERα B domain, namely the motif Ser-102, -104, and -106 and Ser-118. To identify the sites phosphorylated by GSK-3, we generated a series of phosphorylation site-specific ERα mutants in which the serine residues 102, 104, and 118 were mutated to alanine (S102A, S104A, S118A) and a triple mutant in which the serines 102, 104, and 106 were all replaced by alanine (S102A,S104A,S106A). We further generated GST-ERα fusion proteins containing the amino acid residues 76–176 (GST-ERα-(76–176)) of the wild type ERα and the different ERα mutants described. By means of in vitro kinase assays we tested GSK-3 for its ability to phosphorylate these fusion proteins (Fig. 4D). Phosphorylation of the S118A mutant was only slightly weaker compared with the wild type (GST-ERα WT-(76–176)), whereas phosphorylation of the S102A, S104A, and S102A,S104A,S106A mutants was markedly weaker compared with the wild type. Comparison of S102A and S104A phosphorylation revealed a weaker signal in the S104A mutant, indicating that Ser-102 is not phosphorylated if Ser-104 is mutated. This phosphorylation pattern reflects a sequential phosphorylation from the C to the N terminus of the protein that is characteristic for GSK-3. The findings are in agreement with a study that described the disappearance of four phosphorylation sites with mutations of Ser-104 and Ser-106 in the ERα (32). Importantly, the phosphorylated S102A mutant and to a higher degree the S104A and S102A,S104A,S106A mutants showed an upshift in migration compared with the wild type and the S118A mutant (Fig. 4D). This upshift is characteristic for Ser-118 phosphorylation (33), indicating that Ser-118 can be phosphorylated in these mutants by GSK-3β. The weak signal in 32P incorporation observed with the S102A,S104A,S106A mutant agrees with the slightly lower phosphorylation of the S118A mutant observed in comparison with the wild type.

FIGURE 2. GSK-3 interacts with ERα. A, lysates from E2 (10⁻⁷ M)-stimulated cells were immunoprecipitated (IP) with anti-GSK-3α/β or non-immune IgG followed by immunoblotting (IB) with anti-ERα and anti-GSK-3β. B, lysates were immunoprecipitated with anti-ERα or with non-immune IgG followed by immunoblotting with anti-GSK-3β and anti-ERα. C, co-localization (yellow, resulting from close association of red and green fluorescence) of ERα (red) and GSK-3α/β (green); left, untreated; right, E2-treated (10⁻⁷ M, 20 min). Size bar, 20 μm.

FIGURE 3. GSK-3 interacts with the B domain of ERα. A, schematic representation of ERα deletion mutants generated by PCR. B, COS-1 cells were co-transfected with GSK-3β and FLAG-tagged wild type ERα or ERα deletion mutants (ΔF, ΔA, ΔAB). 48 h hours after transfection cells were lysed, and GSK-3 was immunoprecipitated with anti-GSK-3α/β. The lysates (upper panel) and immunoprecipitates (IP) were immunoblotted (IB) with anti-FLAG M2, and GSK-3 amounts in the immunoprecipitates were probed with anti-GSK-3β (lower panel).
Phosphorylation of ERα by GSK-3

Next we studied the impact of the four putative GSK-3 phosphorylation sites on ERα transcriptional activity in vitro. Expression of full-length ERα carrying the different mutations described in ERα-negative MDA-MB231 cells resulted in reduction of E2-induced luciferase activity (Fig. 4E). Similar results were obtained after supertransfection of MELN cells with the ERα mutants (Supplemental Fig. 1). Mutation of any of the serine residues resulted in reduction of ERα activity, which indicates that all serine residues determined as GSK-3 phosphorylation sites in vitro were required for full transcriptional activation of ERα in vivo. This agrees with the findings of other groups using different cell systems (6, 9).

**ERα Phosphorylation by GSK-3 in Vivo**—The phosphorylation state of the serine residues, which had been identified as the GSK-3 phosphorylation sites in vitro, was studied in lysates of MELN cells by immunoblotting using antibodies specific to ERα phosphorylated at Ser-118 or Ser-104 and -106. Ser-104 and -106 were found phosphorylated in unstimulated cells (Fig. 5A). E2 treatment resulted in a strong decrease of phosphorylation of Ser-104 and -106 within 5–30 min. Concomitantly, a marked increase in Ser-118 phosphorylation was observed. These data indicate that the function of the Ser-102, -104, and -106 motif in ERα is different from that of Ser-118.

The involvement of GSK-3 in the phosphorylation of Ser-104 and -106 and Ser-118 of ERα in vivo was studied using the GSK-3 inhibitor LiCl. Treatment of MELN cells with LiCl resulted in inhibition of E2-induced Ser-118 phosphorylation and in reduction of Ser-104 and -106 phosphorylation in both unstimulated cells and in E2-stimulated cells (Fig. 5B). This suggests, on the one hand, Ser-118 phosphorylation by GSK-3 upon E2 treatment. On the other hand hypophosphorylation at the Ser-104 and -106 site caused by LiCl in cells not stimulated with E2 indicates that GSK-3 phosphorylates ERα in unstimulated cells. Similar results were obtained using ERα-positive LNCaP cells (not shown). The effects of LiCl on phosphorylation of cyclin D1 and β-catenin, two well known GSK-3 substrates (29, 30) and transcription factors, were studied for control in the same cell lysates as used for detection of ERα phosphorylation. Treatment with LiCl caused a significant reduction of phosphorylation of both GSK-3 substrates (Fig. 5B). Our observations permit the conclusion that the two serine motifs in the ERα AF-1 domain are regulated differently by GSK-3, which suggests a dual function of GSK-3 in the regulation of ERα activation.

GSK-3 may be regulated by multiple signaling pathways including the PI3K/Akt pathway and the extracellular-regulated kinase pathway. The potential involvement of these pathways in E2-related phosphorylation of GSK-3 was studied by incubation of the cells with a PI3K inhibitor wortmannin and the MEK inhibitor PD98059 (Fig. 5C). E2-induced GSK-3β phosphorylation was completely inhibited by wortmannin but to a low extent by PD98059. This suggests a pronounced role of PI3K and probably the Akt/protein kinase B pathway in GSK-3β phosphorylation. To discriminate the contribution of extracellular-regulated kinase and GSK-3β to E2-induced Ser-118 phosphorylation of ERα, we investigated Ser-118 phosphorylation in the same lysates. We found inhibition of Ser-118 phosphorylation by LiCl but not by PD98059. This
observation adds another argument for Ser-118 phosphorylation by a nuclear pool of GSK-3.

Prolonged treatment of cells with LiCl (72 h) resulted in loss of ERα/H9251 protein (not shown). We hypothesized that destabilization of the protein may be due to dephosphorylation of Ser-102, -104, and -106 as a consequence of GSK-3 inhibition. This assumption was confirmed by co-transfection of wild type GSK-3/H9252 with wild type ERα/H9251 or with the ERα/S102A,S104A,S106A mutant, which cannot be phosphorylated in COS-1 cells. We observed a loss of the mutant ERα/H9251 protein (Fig. 5D).

Mutation of Ser-9 to Alanine in GSK-3/H9252 Prevents Ser-118 Phosphorylation of ERα—The phosphorylation pattern of Ser-104 and -106 and Ser-118 shown in Fig. 5A suggests that dephosphorylation of Ser-104 and -106 is a prerequisite for ERα/H9251 phosphorylation on Ser-118. We, therefore, investigated whether the phosphorylation/inactivation of

FIGURE 5. Sequential phosphorylation of serine residues at two regulatory sites of ERα by GSK-3 is essential for ERα action. A, time dependence of E2 treatment (10^{-7} M) on Ser-104 and -106 and Ser-118 phosphorylation (p) of ERα detected by immunoblotting (IB) with phosphospecific antibodies in cell lysates. B, influence of LiCl on phosphorylation of ERα. Cells were pretreated with LiCl (3 x 10^{-2} M) for 10 min and stimulated with E2 (10^{-7} M) for 20 min. ERα phosphorylation at Ser-104 and -106 and Ser-118 was detected in cell lysates by immunoblotting. The effect of LiCl on phosphorylation of the GSK-3 substrates cyclin D1 and β-catenin was studied for control in the same lysates. C, MELN cells were incubated for 10 min with LiCl (3 x 10^{-2} M), wortmannin (2 x 10^{-7} M), or PD98059 (5 x 10^{-5} M) and thereafter with E2 (10^{-7} M, 20 min). ERα phosphorylation and GSK-3β phosphorylation were detected by immunoblotting. D, COS-1 cells were co-transfected with wild type GSK-3β and wild type ERα or with the S102A,S104A,S106A mutant of ERα. After 72 h of incubation, proteins were detected by immunoblotting. E, MELN cells transiently transfected with FLAG-tagged wild type GSK-3β (GSK-3βWT), mutant GSK-3β/9A, or with empty vector were stimulated with E2 (10^{-7} M, 20 min). ERα phosphorylation was detected in cell lysates. F, MELN cells transiently transfected with empty vector, GSK-3βWT, or GSK-3β9A were stimulated with E2 (10^{-7} M, 20 min). ERE-dependent gene expression was quantified by measuring luciferase activity. -Fold induction is the ratio between stimulated and unstimulated cells.
Phosphorylation of ERα by GSK-3

FIGURE 6. Model for the potential function of GSK-3 in ERα activation. In unstimulated cells (ERα phosphorylated (p) at Ser-102, -104, and -106 is stabilized by interaction with active GSK-3β in the cytoplasm. Treatment of cells with E2 results in phosphorylation of GSK-3β. ERα dissociates from phosphorylated GSK-3β and is dephosphorylated at Ser-102, -104, and -106. ERα translocates into the nucleus and binds to active nuclear GSK-3β where ERα is phosphorylated at Ser-118. Binding of cytoplasmic ligand-bound ERα to the membrane is hypothetical, whereas activation of PI3K by ligand-bound ERα has been suggested in Refs. Castoria et al. (12) and Simoncini et al. (49). Black arrows, reactions shown in this study; open arrows, clarification of the role of reactions in ERα activation needs further investigations. ERK, extracellular signal-regulated kinase; PKB, protein kinase B.

GSK-3 observed in E2-stimulated cells (Figs. 1, B and C and 5C) is a necessary step to achieve dephosphorylation of Ser-104 and -106 and phosphorylation of Ser-118. A mutant of GSK-3β (GSK-3βS9A) that cannot be inactivated was overexpressed in MELN cells. After stimulation with E2, Ser-104 and -106 were not dephosphorylated, and Ser-118 was not phosphorylated in these cells (Fig. 5E). Furthermore, the expression of this mutant resulted in the loss of transcriptional activation of ERα by E2 (Fig. 5F). It is concluded that (transient) inactivation of GSK-3 is necessary for ERα activation. The results from these experiments show that both active and inactive GSK-3 are required for the regulation of ERα function.

Model of a Dual Function of GSK-3 in ERα Action—We propose a new model for the regulation of ERα activation (Fig. 6). GSK-3 phosphorylates ERα at Ser-104 and -106 and probably Ser-102 in unstimulated cells. Under these conditions the interaction of GSK-3 and ERα stabilizes the ligand-free ERα in the cytoplasm. Hormone binding to ERα induces phosphorylation of cytoplasmic GSK-3β probably by activation of the Akt/protein kinase B pathway and dissociation of ERα from GSK-3. Release of the ERα from GSK-3 permits dephosphorylation at Ser-102, -104, and -106, which results in nuclear translocation and in a conformational change of ERα, permitting exposure of Ser-118 to phosphorylation by another protein kinase, most likely a nuclear pool of active GSK-3.

DISCUSSION

In this report we identified ERα as a new GSK-3 substrate that is phosphorylated at four serine residues located within two different motifs in the AF-1 domain of the receptor. We demonstrated functional links between GSK-3 activity and ligand-dependent ERα activation and provided evidence that GSK-3 modulates ERα function by phosphorylation of relevant serine residues.

The serine residues 104, 106, and 118 of ERα, identified as targets for GSK-3 in this study, have been previously attributed to roles in ERα function, Ser-118 representing the major site phosphorylated in response to estradiol (34). Controversy exists, however, regarding the protein kinases that phosphorylate Ser-118 in vivo. A study describing phosphorylation on Ser-118 in ERα expressed in COS-1 cells by mitogen-activated protein kinase (MAPK) (9) contrasts with a report that Ser-118 is phosphorylated upon ligand binding to the receptor in a MAPK-independent manner (33). The latter study explicitly excludes Ser-118 phosphorylation by mitogen-activated protein kinase in MCF-7 cells and postulates a constitutively active protein kinase to phosphorylate this site. Observations that treatment with LiCl results in activation of mitogen-activated protein kinase in intestinal cells and L6 cells (35, 36) but in inhibition of ERα Ser-118 phosphorylation in MELN cells further suggest that other protein kinases may phosphorylate Ser-118 in vivo. Ser-118 is flanked by a proline residue at position 119 of ERα, and this serine-proline motif may represent a non-primed substrate motif for GSK-3. We suggest Ser-118 as a target of GSK-3 by mutational and functional analysis. Mutation of Ser-118 to alanine resulted in a slight decrease in phosphorylation in in vitro kinase assays, and most importantly, an upshift in electrophoretic mobility that is due to Ser-118 phosphorylation (33) was observed in in vitro kinase assays after mutation of other serine residues. Ser-118 phosphorylation in vivo, which was observed in nuclei only (data not shown), was inhibited by the most commonly used GSK-3 inhibitor LiCl but not by the MEK inhibitor PD98059. Furthermore, the kinetics of estradiol-induced Ser-118 phosphorylation correlated with that of ERα and GSK-3 interaction.

Regarding Ser-102, Ser-104, and Ser-106 phosphorylation, ERα sequence analysis suggested that Ser-104 and Ser-106, both flanked by downstream proline residues, represent typical GSK-3 phosphorylation sites. Ser-102 is at four amino acids distance from Ser-106 making Ser-102 a target for GSK-3 if Ser-106 is phosphorylated. Ser-102 phosphorylation on ERα documented in this report has not been reported previously. Phosphopeptide mapping experiments had, however, suggested phosphorylation of other serine residues in the vicinity of Ser-104 and Ser-106 (32). Most GSK-3 substrates require primed phosphorylation, and Ser-106 could represent a target for a priming kinase. However, previous work showed that primed phosphorylation is dispensable when a proline residue is downstream of the targeted amino acid residue, as is the case for Ser-106, and when GSK-3 forms complexes with its substrate (37), as is the case for ERα. Thus, Ser-106 flanked by Pro-107 may represent a motif for direct phosphorylation by GSK-3. This assumption is supported by the reduced phosphorylation signal obtained in in vitro kinase assays using the S102A,S104A,S106A mutant and by the finding that Ser-106 is hypophosphorylated in cells treated with the GSK-3 inhibitor LiCl. The same is true for Ser-104. The phosphorylation of Ser-102, Ser-104, Ser-106, and Ser-118 by GSK-3 shown in this study suggests that, although phosphorylation of these serines may be regulated differently, it may be due to the action of a single kinase. This agrees with the hypothesis proposed by Lannigan (7) that phosphorylation of Ser-118 appears to be regulated differently from that of Ser-104 and Ser-106 and that phosphorylation may be due to the action of a single kinase.

GSK-3 phosphorylates and regulates a large number of transcription factors in either a positive (e.g. CREB (28), MITF (38), P35 (39, 40), and NFκB (19)) or more frequently in a negative manner (e.g. c-Jun (41), c-Myc, C/EBPβ, β-catenin (see Ref. 17), glucocorticoid receptor (42), and androgen receptor (43–45)). A dual function of GSK-3 has been suggested regarding NFκB where phosphorylation at two different sites results either in stabilization or in proteolytic degradation (46).

Our data suggest a new role of GSK-3 as a protein kinase that modulates ERα activity. We show that Ser-102, Ser-104, and Ser-106 are regulated together and independently from Ser-118 by GSK-3, pointing to different regulatory functions of the two phosphorylation sites. Ser-
and Ser-106 phosphorylation is constitutive in ERα-positive cells kept under steroid-deprived conditions. We assume that this is also true for Ser-102, although phospho-Ser-102 could not be demonstrated in cell lysates because of lack of a specific antibody. Functional studies using the respective serine/alanine mutants showed that Ser-102, Ser-104, and Ser-106 have a similar role in ERα transcriptional activation. Phosphorylation of these serine residues seems to contribute to the stability of ligand-free ERα essential for estradiol binding and, therefore, represents the initial step in ERα activation. Inhibition of constitutive ERα phosphorylation by LiCl resulted in loss of ERα protein (not shown), which suggests that ERα not phosphorylated at Ser-102, -104, and -106 is not bound to GSK-3 and down-regulated. This finding supports the assumption of a stabilizing function of GSK-3 on ERα protein. Dephosphorylation of Ser-102, Ser-104, and Ser-106 is a necessary step to achieve Ser-118 phosphorylation, which is the second important function of GSK-3 in ERα activation. The similar reduction of ERα activity observed by mutation of any of these serine residues permits the conclusion that the conformation of ERα, which is disturbed by these mutations, is of great importance for the ordered sequence of phosphorylation of the two regulatory sites by GSK-3.

Complex formation with its substrates is a characteristic feature of GSK-3 which warrants that only a specific pool of GSK-3 related to a specific signaling pathway is activated or inactivated upon a specific stimulus. For example, GSK-3 linked to Wnt signaling is not inhibited by insulin signaling. Furthermore, subcellular distribution to the cytosol, mitochondria, and nuclei (see 16) enables local separation of GSK-3 functions. Our data show that interaction of GSK-3 with the substrate ERα may occur both in the cytoplasm and in nuclei. The different phosphorylation pattern of GSK-3 observed in cytoplasm and nuclei indicates that the enzyme is regulated differently in different cellular compartments, which adds another factor to the complex regulation of GSK-3. Our data show that E2-induced phosphorylation of cytoplasmic GSK-3-β/3 can be inhibited by the PI3K inhibitor wortmannin, which suggests a role of the PI3K/Akt pathway in ERα activation in MELN cells. The precise mechanism resulting in activation of PI3K is not known. It is tempting to speculate, however, that E2 binds to a membrane-associated fraction of ERα. Membrane-bound ERα has been suggested to exert rapid non-genomic effects on cell signaling (47). A certain amount of ERα has been previously localized to the membrane fraction in MELN cells (48). E2 binding to membrane-bound ERα results in a rapid activation of the PI3K/Akt pathway (49), which in turn may result in phosphorylation of the cytoplasmic pool of GSK-3-β only, which is bound to ERα and, thus, could permit nuclear ERα translocation.

GSK-3 also has a function in ligand-independent activation of ERα by non-steroidal compounds such as phorbol esters, which activate the protein kinase C pathway and also result in ERα phosphorylation at Ser-118 (34, 48). Work from our group showed that GSK-3 is phosphorylated by protein kinase Cδ (48), suggesting that phosphorylation and inactivation of GSK-3 as a terminal effector kinase by specific upstream signaling pathways represent a more general mechanism of the regulation of ERα function.

Acknowledgments—We thank P. Angel (German Cancer Research Centre) for GSK-3 cDNA and H. Tröster and E. Spiess (German Cancer Research Center) for valuable discussions and advice in immunofluorescence microscopy. We declare that we have no competing financial interests.
Phosphorylation of ERα by GSK-3

man, S. M., and Menter, D. G. (2004) J. Biol. Chem. 279, 19191–19200
44. Wang, L., Lin, H. K., Hu, Y. C. Xie, S., Yang, L., and Chang, C. (2004) J. Biol. Chem. 279, 32444–32452
45. Mazor, M., Kawano, Y., Zhum, H., Waxman, J., and Kypta, R. M. (2004) Oncogene 23, 7882–7892
46. Demarchi, F., Bertoli, C., Sandy, P., and Schneider, C. (2003) J. Biol. Chem. 241, 39583–39590
47. Hall, J., Couse, J. F., and Korach, K. S. (2001) J. Biol. Chem. 276, 36869–36872
48. De Servi, B., Hermani, A., Medunjanin, S., and Mayer, D. (2005) Oncogene 24, 4946–4955
49. Simoncini, T., Hafezi-Moghadam, A., Brazil, D. P., Ley, K., Chin, W. W., and Liao, J. K. (2000) Nature 407, 538–541