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Growth Hormone Regulates Phosphorylation and Function of CCAAT/Enhancer-binding Protein β by Modulating Akt and Glycogen Synthase Kinase-3*

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Growth hormone (GH) regulates transcription factors associated with c-*fos*, including C/EBPβ. Two forms of C/EBPβ, liver-activating protein (LAP) and liver inhibitory protein (LIP), are dephosphorylated in GH-treated 3T3-F442A fibroblasts. GH-induced dephosphorylation of LAP and LIP is reduced when cells are preincubated with phosphatidylinositol 3′-kinase (PI3K) inhibitors. GH activates Akt and inhibits glycogen synthase kinase-3 (GSK-3). Lithium, a GSK-3 inhibitor, increases GH-dependent dephosphorylation of LAP and LIP. Both are *in vitro* substrates of GSK-3, suggesting that GSK-3 inactivation contributes to GH-promoted dephosphorylation of C/EBPβ. Alkaline phosphatase increases binding of LAP homodimers and decreases binding of LIP homodimers to c-*fos*, suggesting that dephosphorylation of C/EBPβ modifies their ability to bind DNA. Both alkaline phosphatase- and GH-mediated dephosphorylation comparably increase binding of endogenous LAP in 3T3-F442A cells. In cells overexpressing LAP and GSK-3, LAP binding decreases, suggesting that GSK-3-mediated phosphorylation interferes with LAP binding. Expression of constitutively active GSK-3 reduced GH-stimulated c-*fos* promoter activity. These studies indicate that PI3K/Akt/GSK-3 mediates signaling between GH receptor and the nucleus, promoting dephosphorylation of C/EBPβ. Dephosphorylation increases binding of LAP complexes to the c-*fos* promoter and may contribute to the participation of C/EBPβ in GH-stimulated c-*fos* expression.

Growth hormone (GH)[1] exhibits a variety of effects on somatic growth, differentiation, and metabolism (1, 2), which often involve changes in gene expression. Analysis of the regulation of gene transcription by GH has provided additional insight into signaling mechanisms between the GH receptor (GHR) and nuclear events regulating transcription (3, 4). Regulation of the expression of the proto-oncogene c-*fos* has served as a useful model for studying regulation of gene expression by GH (5, 6), and has demonstrated diverse effects of GH on the phosphorylation of transcription factors. For example, GH-stimulated tyrosine phosphorylation of STATs and serine phosphorylation of Elk-1 are required for these transcription factors to activate transcription of c-*fos* in response to GH (7–13).

The CCAAT/Enhancer-Binding Proteins (C/EBPs) have recently been shown to participate in GH-regulated transcription of c-*fos* (14). C/EBPs, which include C/EBPα, C/EBPβ, C/EBPδ, C/EBPϵ, and C/EBPζ/CHOP, belong to the bZIP family of transcription factors characterized by a C-terminal dimerization domain (leucine zipper) adjacent to a basic DNA binding domain. The N-terminal region of C/EBPs contains the transcription activation and inhibitory domains. C/EBPβ, the prominent GH-regulated form (14) is present in cells as three alternate translation products. The 32- and 35-kDa forms of C/EBPβ, also known as liver-activating proteins (LAP), are potent transactivators. The 20-kDa form of C/EBPβ, known as liver inhibitory protein (LIP), possesses a truncated transactivation domain and inhibits transcription (15). LIP inhibits the transactivating potential of LAP, even at relatively low molar ratios (15). It has also been demonstrated that overexpression of LIP in hepatocytes overcomes LAP-mediated cell cycle arrest (16). In the adipocyte differentiation program, C/EBPβ appears to play an important role in the transition between cell cycle progression and terminal differentiation (17–19).

GH increases the binding of C/EBPβ and C/EBPδ to the c-*fos* C/EBP site, which lies -295 to -303 bp relative to the transcription start site, and overlaps the c-*fos* serum response element (SRE) (14). GH promotes a rapid and transient dephosphorylation of LAP and LIP in 3T3-F442A cells, in addition to increasing binding of C/EBPβ to the C/EBP site in c-*fos* (14). It is well established that modulation of target gene expression can be achieved through regulation of the phosphorylation state of transcription factors, which can positively or negatively alter their DNA binding affinity and/or their capacity to activate gene transcription (20–25). The focus of the present study was to identify signaling events by which GH regulates the

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[1] The abbreviations used are: GH, growth hormone; GHR, growth hormone receptor; STAT, signal transducers and activators of transcription; C/EBP, CCAAT/enhancer-binding proteins; LAP, liver-activating protein; LIP, liver inhibitory protein; bp, base pair(s); SRE, serum response element; PI3K, phosphatidylinositol 3-kinase; CHO, Chinese hamster ovary; BSA, bovine serum albumin; MAPK, mitogen-activate protein kinase; AP, alkaline phosphatase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; GSK-3, glycogen synthase kinase-3; CMV, cytomegalovirus; RSV, Rous sarcoma virus; PAGE, polyacrylamide gel electrophoresis; wt, wild-type; PDGF, platelet-derived growth factor; CREB, cAMP-response element-binding protein; AP-1, activator protein-1; SRF, serum response factor.

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dephosphorylation of LIP and LAP and to determine whether dephosphorylation of LAP and LIP modulates their ability to bind to DNA. These studies implicate PI3K/Akt/GSK-3 signaling in the regulation of the dephosphorylation of C/EBPβ by GH, thereby identifying PI3K as a signaling intermediate between the GH receptor and the nucleus. Furthermore, these studies demonstrate that GH-induced dephosphorylation of C/EBPβ dramatically increases binding of LAP complexes to the c-*fos* promoter and may thereby modulate transcriptional activation of c-*fos* by C/EBPβ in response to GH.

EXPERIMENTAL PROCEDURES

Materials—3T3-F442A fibroblasts were provided by Dr. H. Green (Harvard University) and Dr. M. Sonenberg (Sloan-Kettering). Chinese hamster ovary cells expressing rat GHR containing the N-terminal half of the cytoplasmic domain (CHO-GHR) were provided by G. Norstedt (Karolinska Institute, Stockholm, Sweden) and N. Billestrup (Hagedorn Laboratory, Gentofte, Denmark) (26). Human embryonic kidney 293T cells were provided by Dr. M. Lazar (University of Pennsylvania). Recombinant human GH was provided by Eli Lilly. Culture media were purchased from Irvine Scientific. Calf serum and fetal calf serum were purchased from Life Technologies, Inc., albumin (BSA, CRG7) from Intergen. Wortmannin was purchased from Calbiochem. LY294002, lithium chloride, and sodium orthovanadate were purchased from Sigma Chemical Co., and alkaline phosphatase was purchased from Roche Molecular Biochemicals. The MEK inhibitor PD098059 and the GSK-3 peptide substrates were gifts from Dr. A. Saltiel (Pfizer, Ann Arbor, MI). Recombinant GSK-3 was purchased from New England BioLabs. Aprotinin, leupeptin, and Complete® protease inhibitor mixture (EDTA-free) were purchased from Roche Molecular Biochemicals and used according to the supplier's instructions. $[\alpha\text{-}^{32}P]dATP$ and $[\gamma\text{-}^{32}P]ATP$ were from PerkinElmer Life Sciences. The ECL detection system was purchased from Amersham Pharmacia Biotech. Luciferin was purchased from Promega and β-galactosidase chemiluminescence reagents were from Tropix.

Cell Culture and Hormone Treatment—3T3-F442A preadipocytes and 293T cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose and 8% calf serum in an atmosphere of 10% CO_2/90% air at 37 °C. CHO-GHR cells were grown in Ham's F-12 medium containing 10% fetal calf serum and 0.5 mg/ml G418 in an atmosphere of 5% CO_2/95% air at 37 °C. All media were supplemented with 1 mM L-glutamine, 100 units/ml penicillin, 100 mg/μl streptomycin, and 0.25 μg/ml amphotericin. Prior to treatment, cells were incubated overnight in the appropriate medium containing 1% BSA instead of serum, then cells were incubated with or without GH at 500 ng/ml (22 nM) as indicated.

Plasmids and Antibodies—Plasmids encoding LAP or LIP driven by the CMV promoter (CMV-LAP and CMV-LIP) were gifts from Dr. U. Schibler (University of Geneva) courtesy of L. Sealy (Vanderbilt University). The plasmid RSV-β galactosidase (RSV-β-gal) was provided by Dr. M. Uhler (University of Michigan). CMV-GSK-3 S9A was provided by P. J. Roach (Indiana University), and pcDNA3.1 was purchased from CLONTECH. The plasmid wt-*fos*-Luc, provided by Dr. W. Wharton (University of South Florida) (27) contains 379 bp of the mouse c-*fos* promoter immediately 5′ of the transcription start site cloned upstream of the luciferase gene.

Specific rabbit polyclonal antibodies against a peptide corresponding to amino acids 278–295 at the C terminus of C/EBPβ were purchased from Santa Cruz Biotechnology, Inc. Polyclonal antibodies that recognize phosphorylated Ser-473 of Akt (anti-P-Akt), and polyclonal antibodies made against the Akt peptide 466–479 (anti-Akt) were purchased from New England BioLabs. Polyclonal antibodies against an oligopeptide corresponding to amino acids 16–26 of GSK-3α phosphorylated on Ser-21 (anti-P-GSK-3) and to a peptide corresponding to amino acids 203–219 of GSK-3 (anti-GSK-3) were purchased from Upstate Biotechnology Inc.

Immunoblotting—Cell lysis and immunoblotting for C/EBPβ were performed as previously described (14) and for Akt and GSK-3 as follows: confluent 3T3-F442A cells on 100-mm plates were washed with phosphate-buffered saline with vanadate (10 mM Tris (pH 7.4), 150 mM sodium phosphate, 1 mM sodium vanadate) and scraped in 0.3 ml of L-RIPA lysis buffer (50 mM Hepes, pH 7.0, 250 mM NaCl, 0.5% Triton X-100) containing 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin and leupeptin. Whole cell lysates (35–50 μg) were analyzed by immunoblotting using antibodies against phospho-Akt or phospho-GSK-3. Blots were reprobed as previously described (8) using the corresponding antibody against Akt or GSK-3. The apparent M_r are based on prestained molecular weight standards (Life Technologies, Inc.).

GSK-3 Activity—3T3-F442A cells were washed with phosphate-buffered saline and lysed in GSK-3 extraction buffer (50 mM Hepes (pH 7.4), 1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 5 mM Na_2PO_4, 100 mM KCl, 0.5% Triton X-100, 1 mM dithiothreitol, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 mM sodium vanadate). Samples were centrifuged at 15,000 × *g* for 10 min. GSK-3 activity was measured in the supernatant (8 μg of protein) in a final volume of 25 μl of 50 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mg/ml synthetic peptide (RRAAEELDSRAG(p)SPQL) or negative control peptide (RRAAEELDSRAGAPQL), and 100 μM $[\gamma\text{-}^{32}P]ATP$ (1000 cpm/pmol). After incubation for 15 min at 30 °C, the reactions were terminated by the addition of (5 μl) 100 mM EDTA, 5 mM ATP, and tubes were placed on ice. An aliquot (20 μl) of the reaction mixture was spotted onto Whatman p81 phosphocellulose paper, washed in three changes of 175 mM phosphoric acid for a total of 20 min, air-dried, and ^{32}P incorporation was measured by liquid scintillation counting. ^{32}P incorporation into the negative control peptide was subtracted from values obtained using the GSK-3 peptide substrate. Results are expressed as nanomoles of phosphate incorporated per min per mg of protein.

C/EBPβ Immunoprecipitation and in Vitro GSK-3 Phosphorylation Assay—3T3-F442A cells were washed and scraped in RIPA 0.5% SDS buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EGTA, 0.5% SDS). C/EBPβ was immunoprecipitated with an antibody against the C-terminal domain of C/EBPβ (5 μl) (or an equivalent volume of rabbit non-immune serum) for 2 h at 4 °C. After centrifugation, pellets were washed three times with RIPA 0.5% SDS buffer and resuspended in 50 μl of GSK-3 buffer (50 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol). Recombinant GSK-3 (15 units) and $[\gamma\text{-}^{32}P]ATP$ (500 μCi/μmol) were added. After 40 min of incubation at 30 °C, the reaction was stopped by boiling the samples in the presence of sample buffer. The proteins were separated by 15% SDS-PAGE as previously described (14), and phosphorylation was determined by autoradiography. An aliquot of the sample was subjected to immunoblot analysis to identify C/EBPβ in the immunoprecipitate.

Electrophoretic Mobility Shift Assay (EMSA)—Confluent 3T3-F442A cells were deprived of serum overnight and incubated for the indicated times with hormone or vehicle as described above. Nuclear extracts were prepared as described previously (14). Binding reactions proceeded for 30 min at room temperature with labeled oligonucleotides containing wild-type c-*fos* C/EBP site and flanking SRE (wt C/EBP-SRE) or the C/EBP binding site from the *422/aP2* gene (aP2-C/EBP) that were previously described (14). Complexes formed by LAP and/or LIP were separated by nondenaturing 7% PAGE followed by autoradiography. For analysis of LAP or LIP, 293T cells were transfected using calcium phosphate coprecipitation (28) with plasmids CMV-LAP (1 μg) or CMV-LIP (l μg) with or without CMV-GSK-3 S9A (8 μg). 48 h later, cell lysates enriched in nuclear proteins were prepared using high salt buffer (420 mM NaCl, 20 mM Hepes (pH 7.9), 1 mM EDTA, 1 mM EGTA, 50% glycerol, protease inhibitor mixture) and were stored at −80 °C. In some experiments, the enriched lysates were incubated with or without 200 units of alkaline phosphatase, in the presence or absence of orthovanadate (30 mM), for 1 h at 37 °C prior to EMSA or immunoblotting.

Gene Expression Assay—CHO-GHR (1×10^5 cells/35-mm well) were transiently transfected by calcium phosphate coprecipitation (28) with wt-*fos*-Luc (0.4 μg) and the RSV β-galactosidase plasmid (0.1 μg), with or without a plasmid encoding a constitutively active GSK-3 (GSK-3 S9A 0.8 μg), in the presence or absence of CMV-LAP DNA (1 ng) or corresponding amounts of pcDNA3.1 vector per 35-mm well. Twenty-four hours after transfection, cells were deprived of serum by incubation in medium containing 1% BSA for 18 h prior to treatment as indicated. Cell lysates were prepared in reporter lysis buffer (100 mM potassium phosphate, 0.2% Triton X-100, 1 mM dithiothreitol), and luciferase or β-galactosidase activity was measured using an Opticomp luminometer. The luciferase values were normalized to β-galactosidase activity. Each condition was tested in duplicate or triplicate in each experiment. Analysis of variance with factorial Scheffe *F* test was used to analyze data as indicated.

RESULTS

GH-induced Dephosphorylation of LAP and LIP Is Blocked by PI3K Inhibitors—GH causes a rapid and transient dephosphorylation of C/EBPβ, as indicated by an increased mobility

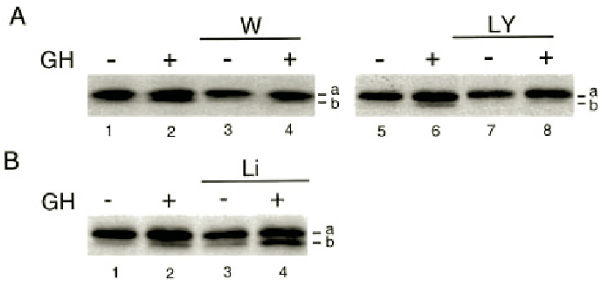

FIG. 1. **Inhibition of PI3K reduces GH-induced dephosphorylation of LIP.** *A*, 3T3-F442A fibroblasts were incubated with wortmannin (*W*, 200 nM) (*lanes 3* and *4*), LY294002 (*LY*, 10 μM), or respective vehicles (*lanes 1–2* and *5–6*) for 30 min prior to treatment without (−) or with (+) GH for an additional 60 min. The cells were lysed and used for immunoblotting with anti-C/EBPβ (1:1000). Bands representing LIP (20 kDa) are shown, and the slower (*a*) and faster (*b*) migrating forms of LIP are indicated. Data are representative of four independent experiments. Similar changes were observed for LAP (not shown) in each experiment. *B*, 3T3-F442A cells were incubated with LiCl (*Li*, 25 mM) for 30 min prior to treatment without (−) or with (+) GH for additional 60 min. The cells were lysed and analyzed by immunoblotting as indicated in part *A*. Data are representative of four independent experiments. Similar results were observed for LAP (not shown) in each experiment.

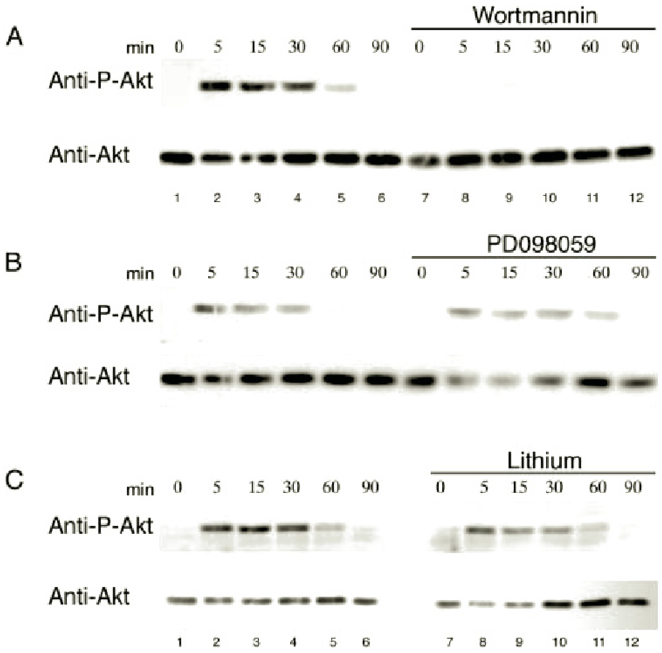

FIG. 2. **GH transiently activates Akt.** 3T3-F442A cells were treated without or with (*A*) wortmannin (200 nM), (*B*) PD098059 (40 μM), or (*C*) LiCl (25 mM) for 30 min. Then GH (*lanes 2–6*, *8–12*) or vehicle (*lanes 1*, *7*) were added for the times indicated. Cells lysates were immunoblotted with anti-P-Akt (1:1000) (*upper panels*). Blots were stripped and reprobed with anti-Akt (1:1000) (*lower panels*). Similar findings were obtained in two independent experiments.

on SDS-PAGE (14). GH is known to activate PI3K and MAPKs ERK1 and ERK2 in several cell types, including 3T3-F442A fibroblasts (29–33). To evaluate whether either of these signaling pathways mediates GH-regulated dephosphorylation of LAP and LIP, we tested the effect of PI3K or MEK inhibitors on the ability of GH to promote dephosphorylation of LAP and LIP. Treatment of the cells with the PI3K inhibitor wortmannin (30 min) prior to treatment with GH (60 min) markedly impaired the GH-promoted dephosphorylation of LIP (Fig. 1*A*, *band b*, *lane 4 versus lane 2*) and LAP (not shown). Similar inhibition of GH-promoted dephosphorylation was obtained when cells were treated with LY294002, another PI3K inhibitor, prior to GH (Fig. 1*A*, *band b*, *lane 8 versus lane 6*). Taken together these results suggest a role for PI3K in the GH-promoted dephosphorylation of C/EBPβ. Treatment with the MEK inhibitor PD098059 (40 μM, 30 min) prior to GH did not interfere with the GH-promoted dephosphorylation of LIP and LAP (not shown), indicating that the MEK-ERK pathway is unlikely to be a major contributor to this response to GH. Similar changes in phosphorylation in response to GH were observed for LAP and LIP throughout these studies.

Because activation of PI3K is known to lead to phosphorylation and inactivation of GSK-3, involvement of GSK-3 in GH-induced dephosphorylation of C/EBPβ was examined. Cells were pretreated with LiCl, a GSK-3 inhibitor (34, 35), which slightly increased the intensity of the rapidly migrating LIP band in control cells (Fig. 1*B*, *band b*, *lane 3*). Notably, the GH-dependent shift to the rapidly migrating form of LIP was further enhanced in the presence of LiCl (Fig. 1*B*, *band b*, *lane 4 versus lane 2*), suggesting that inhibition of GSK-3 favors the presence of the dephosphorylated form of C/EBPβ. Treatment with LiCl increased dephosphorylation of LAP similarly to the increase observed for LIP in GH-treated and untreated cells. In contrast, neither wortmannin nor LiCl altered the pattern of phosphorylation of ERK-1 or -2 in the absence or presence of GH (data not shown), indicating that wortmannin and LiCl do not affect the MAPK pathway under the conditions of these experiments. These data are consistent with GH utilizing a PI3K pathway to modulate dephosphorylation of C/EBPβ and suggest that GSK-3 may be involved downstream of PI3K.

GH Activates Akt—Akt is a downstream effector of PI3K (36–38). The ability of GH to stimulate phosphorylation of Akt was examined by immunoblotting, using an antibody that specifically recognizes a peptide of Akt phosphorylated on Ser-473 (anti-P-Akt). GH was found to increase the amount of Ser-phosphorylated Akt within 5 min (Fig. 2, *A*, *B*, and *C*, *upper panels*, *lanes 1*, *2*). The stimulation of Akt by GH subsided from 15 to 60 min (Fig. 2, *A*, *B*, and *C*, *upper panels*, *lanes 3–6*). The lower panels show total Akt. The PI3K inhibitor wortmannin completely blocked the GH-stimulated activation of Akt (Fig. 2*A*, *lanes 7–12*), consistent with GH-mediated activation of Akt being dependent on PI3K. When 3T3-F442A cells were pretreated with PD098059, the appearance of phosphorylated Akt in response to GH was not altered (Fig. 2*B*, *lanes 7–12*), suggesting that the MEK-ERK pathway is not involved in GH activation of Akt. Lithium, a GSK-3 inhibitor, also failed to alter Akt phosphorylation in response to GH (Fig. 2*C*, *lanes 7–12*), as expected, because GSK-3 lies downstream of Akt. Taken together, these results indicate that, in 3T3-F442A cells, GH promotes phosphorylation and activation of Akt downstream of PI3K.

GH Inhibits GSK-3—GSK-3 activity is inhibited by growth factors that activate PI3K and Akt in several cell types (39–41). GSK-3 is inactivated when it is phosphorylated downstream of Akt (39). Hence, it would be predicted that activation of Akt by GH would be associated with inhibition of GSK-3. The activity of GSK-3 was measured by its ability to phosphorylate a synthetic peptide containing a GSK-3 consensus sequence in the presence of [γ-^{32}P]ATP (42). Treatment of 3T3-F442A cells with GH resulted in a 30–40% inhibition of GSK-3 activity within 15 min (Fig. 3*A*). The GH-mediated inhibition of GSK-3 was transient, and GSK-3 activity returned to baseline within 30 min.

Akt-dependent phosphorylation of the GSK-3α isoform on Ser-21 or of GSK-3β on Ser-9 results in partial inactivation of the kinase (43). Consistent with GH-mediated inactivation of GSK-3, GH was found to increase the amount of serine-phosphorylated GSK-3, as detected by immunoblotting in lysates from 3T3-F442A cells with an antibody that specifically recog-

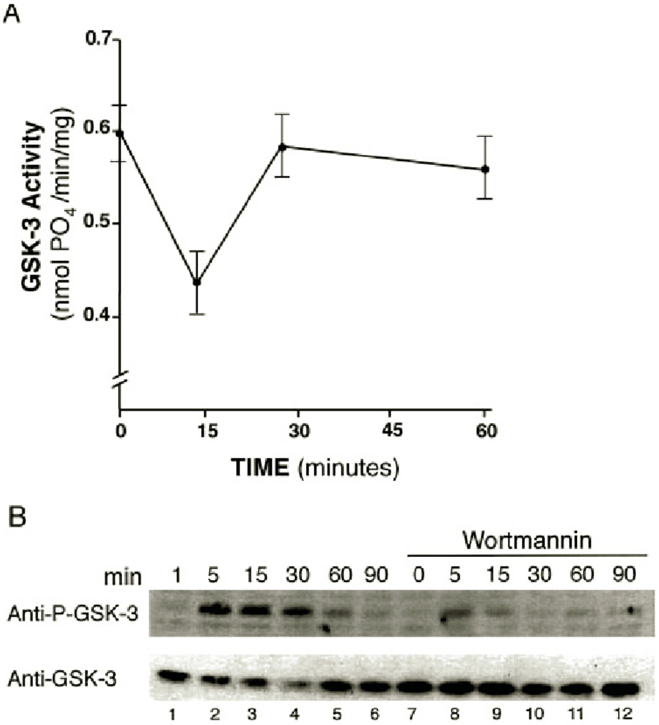

FIG. 3. **GH transiently inhibits GSK-3 activity.** *A*, 3T3-F442A cells, treated with or without GH (500 ng/ml) for the indicated times, were lysed, and GSK-3 activity was measured using a synthetic peptide and [γ-^{32}P]ATP. Data shown are the average ± S.E. of three independent experiments. *B*, 3T3-F442A cells were treated without or with wortmannin (200 nM) for 30 min prior to incubation with GH for the times indicated. Cells were lysed and analyzed by immunoblotting with anti-P-GSK-3 (1:1000). The blots were stripped and reprobed with anti-GSK-3 (1:1000). The immunoblots are representative of three independent experiments.

nizes phosphorylated Ser-21 of GSK-3α. The increase was evident within 5 min of GH treatment (Fig. 3*B*, *lane 2*), and subsided progressively (15–90 min)(Fig. 3*B*, *lanes 3*-6). As expected, pretreatment with wortmannin reduced the GH-induced phosphorylation of GSK-3 (Fig. 3*B*, *lanes 7–12 versus 1–6*). These results indicate that GH stimulates phosphorylation and inhibition of GSK-3 most likely by a mechanism involving PI3K.

LAP and LIP Are Substrates of GSK-3 in Vitro—Because inhibition of GSK-3 by GH might contribute to the ability of GH to promote dephosphorylation of LAP and LIP, and because both LAP and LIP contain a putative consensus sequence for GSK-3, the possibility that LAP and/or LIP are GSK-3 substrate(s) was tested. LAP and LIP were immunoprecipitated from 3T3-F442A cells and incubated with [γ-^{32}P]ATP in the presence or absence of recombinant GSK-3. When anti-C/EBPβ immunoprecipitates were incubated with GSK-3 in the presence of [γ-^{32}P]ATP, autoradiography revealed prominent phosphorylated proteins (Fig. 4*A*, *lane 4*) with the sizes appropriate for LAP and LIP (Fig. 5*B*, *lane 6*). No phosphoproteins were detected when the incubation was performed in the absence of GSK-3 (Fig. 4*A*, *lane 2*) indicating that other kinase activity did not co-precipitate with C/EBPβ. When non-immune serum was used instead of anti-C/EBPβ, labeled proteins were not detected in the absence or presence of GSK-3 (*lanes 1* and *3*). These results indicate that *in vitro* LAP and LIP are substrates of GSK-3.

Dephosphorylation of LAP Increases DNA Binding—The effect of dephosphorylation of LAP and LIP on their DNA binding capacity was investigated for insight into the functional importance of the phosphorylation state of C/EBPβ. Extracts from 293T cells (which lack detectable C/EBPβ) overexpressing LAP or LIP were subjected to EMSA to detect LAP and LIP complexes bound to the c-*fos* C/EBP site and overlapping SRE (wtC/EBP-SRE) as probe. When a combination of extracts each enriched in LAP or LIP was subjected to EMSA, homodimers of LAP (*upper complex*) and LIP (*bottom complex*), and heterodimers of LAP and LIP (*middle band*) were detected (Fig. 5*A*, *lane 1*). The addition of antibodies specific for C/EBPβ caused a supershift of these complexes (Fig. 5*A*, *lane 2*, *arrow*). A faint band representing endogenous SRF in 293T cells was also observed (Fig. 5*A*, *lane 1*, *top band*).

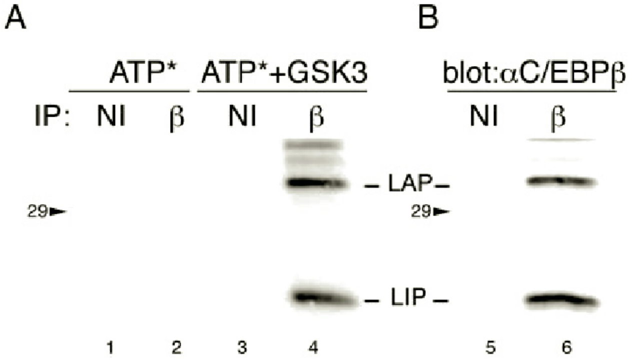

FIG. 4. **LAP and LIP are *in vitro* substrates of GSK-3.** *A*, lysates of 3T3-F442A fibroblasts were immunoprecipitated with anti-C/EBPβ or treated with non-immune serum (*NI*). Immune complexes were incubated with [γ-^{32}P]ATP (*ATP**) in the absence (*lanes 1*, *2*) or presence of GSK-3 (*lanes 3*, *4*). *B*, aliquots of the same samples as in *A* were incubated with non-immune serum (*lane 5*) or anti-C/EBPβ (*lane 6*) and immunoblotted with anti-C/EBPβ (1:1000). Data are representative of two independent experiments.

To examine the effect of dephosphorylation on binding to the c-*fos* C/EBP site, extracts enriched in LAP were incubated with alkaline phosphatase (AP) and subjected to EMSA. The resulting dephosphorylation of LAP caused a dramatic increase in binding of LAP homodimers (Fig. 5*B*, *lane 2 versus lane 1*). The increase in binding by alkaline phosphatase was prevented by the simultaneous addition of the phosphatase inhibitor orthovanadate to the incubation medium (data not shown). The dephosphorylation of LAP by alkaline phosphatase was verified by immunoblotting (Fig. 5*C*), showing that alkaline phosphatase treatment caused LAP to migrate more rapidly (*lane 2 versus lane 1*), an effect that was prevented by orthovanadate (data not shown). Interestingly, alkaline phosphatase treatment of extracts enriched with LIP led to a decrease in binding of LIP homodimer to the C/EBP site (Fig. 5*B*, *lane 4 versus lane 3*). Alkaline phosphatase treatment caused LIP to shift to a faster mobility form on immunoblots (Fig. 5*C*, *lane 4 versus lane 3*) consistent with LIP dephosphorylation. Thus, dephosphorylation has opposite effects on the ability of LAP and LIP to bind as homodimers to the C/EBP site of the c-*fos* promoter, increasing binding of LAP homodimers and decreasing binding of LIP homodimers.

When alkaline phosphatase-treated extracts enriched in LAP and LIP were combined, dephosphorylation increased binding of LAP homodimers (Fig. 5*B*, *lane 6 versus lane 5*) and decreased binding of LIP homodimers (detectable in longer autoradiographic exposures, not shown) similar to the changes observed when LAP and LIP were tested alone. Interestingly, binding of LAP/LIP heterodimers was increased by dephosphorylation, suggesting that LAP may recruit LIP for heterodimer formation. Taken together, these results suggest that the phosphorylation status of LAP and LIP plays a key role in their ability to bind as homo- and/or heterodimers to the C/EBP site of the c-*fos* promoter.

To test further the effect of dephosphorylation on the DNA

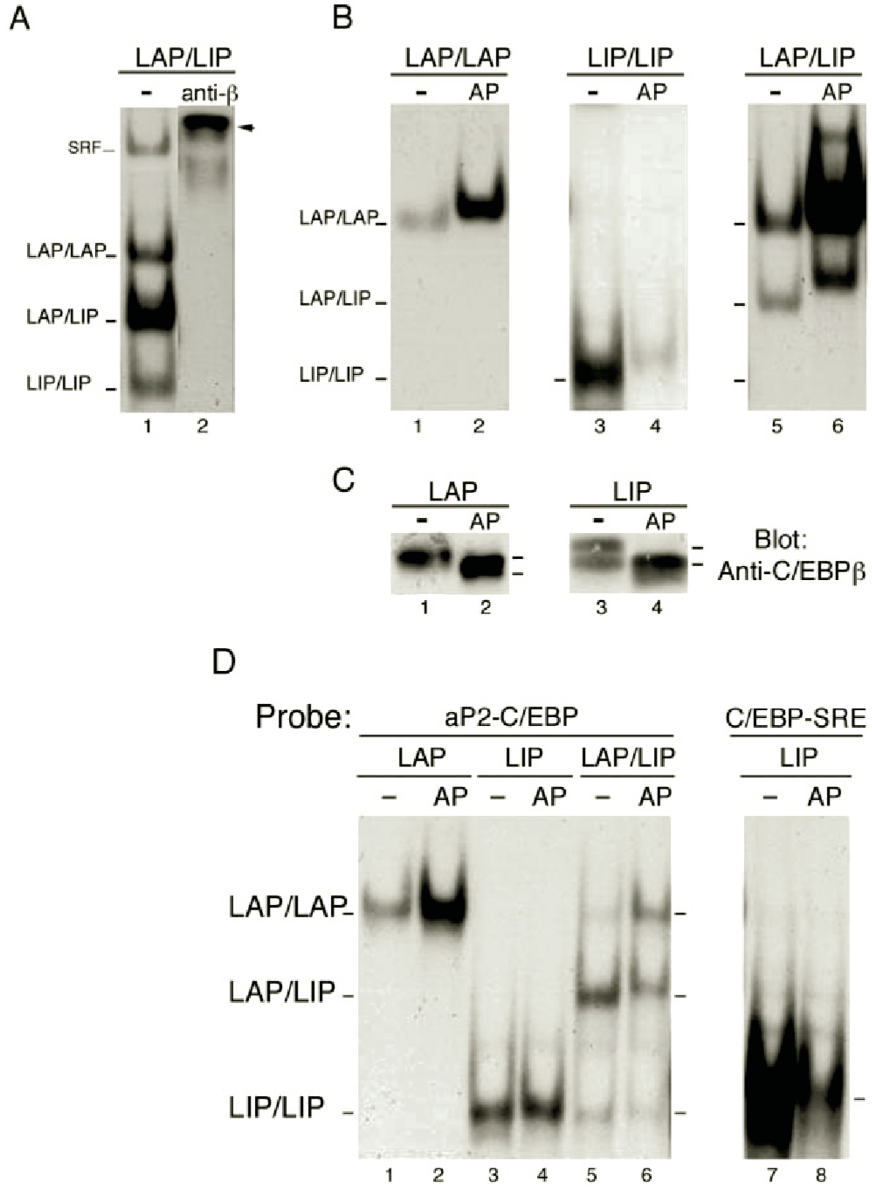

FIG. 5. **Dephosphorylation of LAP, but not LIP, increases DNA binding.** *A*, extracts from 293T cells overexpressing LAP or LIP were mixed and incubated in the absence (*lane 1*) or presence (*lane 2*) of antiserum specific for C/EBPβ (*anti-β*), and complexes containing LAP and/or LIP were identified by EMSA using wt C/EBP-SRE as probe. Migration of complexes representing LAP homodimers (*LAP/LAP*), LIP homodimers (*LIP/LIP*), and LAP/LIP heterodimers (*LAP/LIP*) is shown (*left margin*). The *arrow* (*lane 2*) marks supershift with anti-C/EBPβ. The *faint upper band* in *lane 1* represents endogenous SRF. *B*, extracts from 293T cells overexpressing LAP or LIP were incubated without (*lanes 1*, *3*, *5*) or with alkaline phosphatase (*AP*, 200 units) (*lanes 2*, *4*, *6*). The extracts were incubated separately (*lanes 1–4*) or in combination (*lanes 5–6*) with the wt C/EBP-SRE probe for EMSA. LAP- or LIP-containing complexes are indicated as in *A*. Similar results were obtained in 10 independent experiments. *C*, extracts used in part *B* containing LAP (*lanes 1*, *2*) or LIP (*lanes 3*, *4*) were analyzed by immunoblotting with antibody specific for C/EBPβ after treatment with (*lanes 2*, *4*) or without (*lanes 1*, *3*) alkaline phosphatase. Slowly (phosphorylated) and rapidly (dephosphorylated) migrating forms of LAP and LIP are marked by *upper* and *lower bars*, respectively, in the *right margin*. *D*, extracts enriched in LAP (*lanes 1*, *2*) or LIP (*lanes 3*, *4*, *7*, *8*), with or without alkaline phosphatase treatment, were incubated alone (*lanes 1–4*) or in combination (*lanes 5*, *6*) with a probe containing the C/EBP site of the *aP2/422* gene for EMSA. Binding of the same preparation of LIP to the c-*fos* C/EBP-SRE probe, with and without alkaline phosphatase treatment, is compared in the same experiment (*lanes 7* and *8*). Migration of LAP- and LIP-containing complexes is indicated as in *A*. Similar results were obtained in three independent experiments.

binding capacity of LAP and LIP complexes, a probe based on the C/EBP site from the *422/aP2* gene, which is not associated with the SRE, was tested. Alkaline phosphatase treatment of LAP-enriched extracts increased binding of LAP homodimers (Fig. 5*D*, *lane 2 versus lane 1*), indicating that the increase in binding with dephosphorylation of LAP is likely to be independent of the presence of the SRE sequence. However, dephosphorylation of LIP did not alter binding of LIP homodimers to the aP2-C/EBP probe (Fig. 5*D*, *lane 4 versus lane 3*), in contrast to the decrease obtained with the C/EBP-SRE probe (*lane 7 versus* 8). When alkaline phosphatase-treated extracts enriched in LAP and LIP were combined, dephosphorylation increased binding of LAP homodimers (Fig. 5*D*, *lane 6 versus lane 5*, *upper band*). The binding of LIP homodimers (*bottom band*) was not altered even in longer autoradiographic exposures (not shown), whereas the binding of LAP/LIP dimers (*middle band*) was slightly decreased.

Taken together, these results indicate that *in vitro* dephosphorylation of LAP facilitates its binding to DNA in the absence as well as the presence of the SRE sequence. In contrast, *in vitro* dephosphorylation of LIP decreases binding of LIP homodimers to the c-*fos* C/EBP site but does not modify its binding to the C/EBP site in the aP2 probe, suggesting that dephosphorylation of LIP may have different consequences in the regulation of LIP DNA binding capacity.

Dephosphorylation Facilitates Binding of Endogenous LAP to the c-fos C/EBP Site—To evaluate whether the binding of endogenous LAP and LIP is also altered by dephosphorylation, nuclear extracts from 3T3-F442A cells were incubated with alkaline phosphatase for 1 h and analyzed by EMSA. The resulting dephosphorylation of endogenous LAP and LIP by alkaline phosphatase increased binding of LAP/LAP and LAP/LIP dimers (Fig. 6, *lane 2 versus lane 1*). GH increased the binding of endogenous LAP homodimers and LAP/LIP heterodimers, as reported previously (14). The extent of the increase with GH was comparable to the increase promoted by alkaline phosphatase-mediated dephosphorylation of LAP and LIP in the same experiment (Fig. 6, *lane 2 versus lane 3*). The comparable increases with alkaline phosphatase and GH support the idea that GH-promoted dephosphorylation of endogenous LAP contributes to the ability of GH to increase binding of LAP complexes. The binding of LIP homodimers was not detectable in 3T3-F442A cells under any condition tested. The inability to detect endogenous LIP homodimers may be related to apparent recruitment of LIP to LAP/LIP heterodimers. Dephosphorylation of LAP and LIP was confirmed by immunoblot

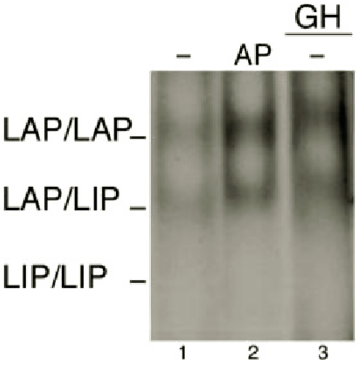

FIG. 6. **Dephosphorylation of endogenous LAP increases its binding to DNA.** Nuclear extracts from 3T3-F442A cells were incubated without (*lane 1*) or with alkaline phosphatase (*AP*, 200 units) (*lane 2*) prior to EMSA with the wt C/EBP-SRE probe. Nuclear extracts from cells incubated with GH for 60 min (*lane 3*) were also analyzed by EMSA. Migration of complexes containing LAP and/or LIP is shown to the *left* and was determined by comparison to LAP- or LIP-enriched extracts from 293T cells on same gel. Similar data were obtained in two independent experiments.

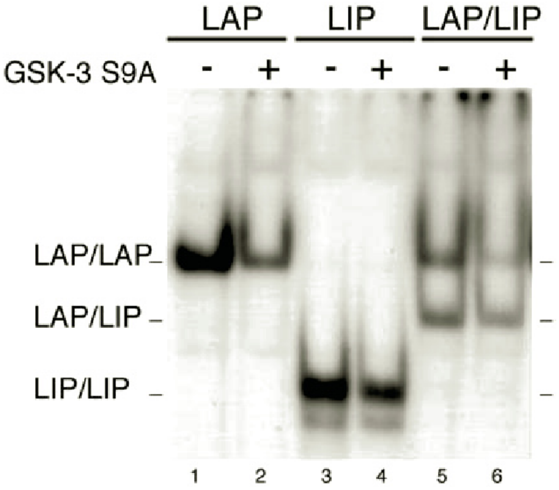

FIG. 7. **GSK-3 impairs the DNA binding capacity of LAP.** LAP or LIP were expressed in 293T cells in the presence or absence of constitutively active GSK-3 (*GSK-3 S9A*). The amount of expressed protein in the extracts was quantified by prior immunoblotting and equivalent amounts of LAP (*lanes 1, 2*) or LIP (*lanes 3, 4*) were tested individually (*lanes 1–4*) or in combination (*lanes 5, 6*) by EMSA with wt C/EBP-SRE. LAP- and LIP-containing complexes are indicated as in Fig. 5. Similar results were obtained in four independent experiments.

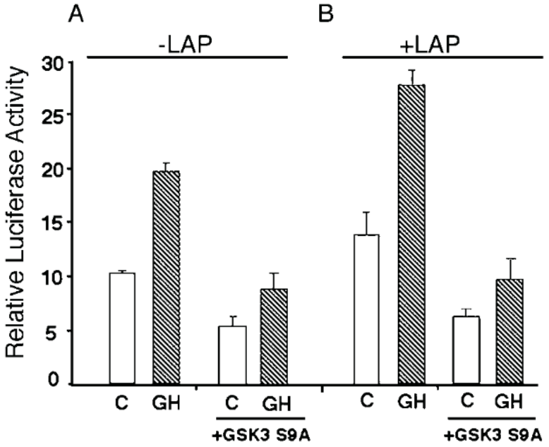

FIG. 8. *A*, **Expression of GSK-3 reduces GH-stimulated *c-fos* promoter activity.** CHO-GHR cells were transiently transfected with CMV-GSK-3 S9A (*+GSK-3 S9A*) or empty vector, with wt-*c-fos*-luc plasmid and RSV-β-gal. After 48 h, cells were treated with GH (*hatched bars*) or vehicle (*open bars*) for 4 h, and luciferase activity was measured and normalized to β-galactosidase activity. Each *bar* represents the mean ± S.E. for six independent experiments. Luciferase activity was significantly different ($p < 0.001$) between control and GH-treated cells in the absence of GSK-3 S9A and between controls in the absence and presence of GSK-3 S9A. There was not a significant difference between control and GH-treated cells in the presence of GSK-3 S9A. *B*, LAP does not overcome GSK-3 restraint of basal or GH-stimulated c-*fos* promoter activity. CHO-GHR cells were transiently transfected with plasmids for c-*fos*-luc, RSV-β-gal, CMV-LAP, and CMV-GSK-3 S9A or empty vector and were treated and analyzed as in part *A*. Each *bar* represents the mean ± S.E. for four independent experiments. The response to GH is significant ($p < 0.04$) in the absence of GSK-3 S9A.

(data not shown). These data suggest that dephosphorylation of endogenous LAP, as promoted by GH, can increase the binding of LAP homodimers and LAP/LIP heterodimers to the c-*fos* promoter.

GSK-3 Restrains LAP Binding to the c-fos C/EBP Site—If dephosphorylation increases binding of LAP complexes to DNA, then it would be predicted that phosphorylation, as mediated by GSK-3, might reduce such binding. To examine whether GSK-3 alters the ability of LAP and/or LIP to bind DNA, LAP or LIP was overexpressed in 293T cells in the presence or absence of constitutively active GSK-3 (GSK-3 S9A). Co-expression of LAP and GSK-3 S9A reduced the binding of LAP homodimers to DNA by almost half (Fig. 7, *lane 2 versus lane 1*). The decrease in LAP binding was also evident when extracts from cells overexpressing LAP or LIP in the presence of GSK-3 S9A were combined; binding of LAP/LAP homodimers, and to a lesser extent of LAP/LIP heterodimers, was reduced in the presence of GSK-3 S9A (Fig. 7, *lane 6 versus lane 5*). Co-expression of LIP and GSK-3 S9A slightly decreased the binding of LIP homodimers (Fig. 7, *lane 3 versus lane 4*). These data suggest that phosphorylation, as mediated by GSK-3, decreases the ability of LAP and LIP to bind as homo- or heterodimers to the C/EBP site of the c-*fos* promoter.

Role of GSK-3 on GH-stimulated c-fos Promoter Activity—To examine whether GSK-3 modulates GH-regulated c-*fos* promoter expression, constitutively active GSK-3 (GSK-3 S9A) was expressed in combination with a luciferase reporter driven by wild type c-*fos* promoter (wt-*fos*-Luc) in GH-responsive CHO-GHR cells. Expression of GSK-3 S9A reduces the basal level of c-*fos* promoter activity by 50% (Fig. 8*A*, *open bars*). Moreover, expression of GSK-3 S9A reduces the ability of GH to stimulate reporter expression via the c-*fos* promoter (Fig. 8*A*, *hatched bars*). The c-*fos* promoter is capable of being stimulated by 10% calf serum to the same level in the presence (5.5- ± 0.6-fold) or absence (5.1- ± 0.5-fold) of GSK-3 S9A. These data indicate that GSK-3 activity restrains c-*fos* expression and specifically interferes with stimulation of c-*fos* by GH.

Overexpression of LAP elevates basal c-*fos* promoter activity with respect to that observed in vector-transfected cells (Fig. 8*B versus* 8*A*, *leftmost open bars*), as reported previously (14). Stimulation of c-*fos* promoter activity by GH is ~2-fold relative to untreated control in the presence or absence of overexpressed LAP (Fig. 8*B versus* Fig. 8*A*, *leftmost hatched bars*), as reported previously (14). To determine whether LAP could overcome the ability of GSK-3 to interfere with GH-induced c-*fos* promoter activity, GSK-3 S9A was co-expressed with LAP and wt-*fos*-Luc. Basal c-*fos* promoter activity was reduced in the presence of GSK-3 S9A despite the presence of LAP (Fig. 8*B*, *open bars*). Furthermore, GSK-3 S9A interfered with GH-stimulated c-*fos* promoter activity in the presence of LAP (Fig. 8*B*, *hatched bars*) as well as the absence of LAP (Fig. 8*A*, *hatched bars*). Taken together, these data indicate that GSK-3 restrains c-*fos* expression-stimulated by GH and suggest that transient inhibition of GSK-3 by GH plays a role in the GH-stimulated expression of c-*fos*. These

data also suggest that GSK-3 activity interferes with the ability of LAP to stimulate the c-*fos* promoter in the absence or presence of GH; such interference could be due in part to the restraining effect of GSK-3 on the binding of LAP dimers to the c-*fos* promoter.

DISCUSSION

GH-stimulated PI3K Signaling Regulates C/EBPβ in the Nucleus—The present studies are consistent with GH utilizing a PI3K-mediated pathway to alter the phosphorylation state of C/EBPβ in the nucleus, by a mechanism involving activation of Akt and inhibition of GSK-3. It has been known for some time that GH can initiate PI3K signaling by stimulating tyrosine phosphorylation of insulin receptor substrate family proteins, and the association of insulin receptor substrates 1 and 2 with PI3K (44–47). This study demonstrates that C/EBPβ is a likely nuclear end point for a GH-stimulated PI3K-mediated pathway, based on reversal of LAP and LIP dephosphorylation by PI3K inhibitors. Thus for the first time, C/EBPβ is identified as a nuclear target for a GH-stimulated PI3K pathway and shows that PI3K may be a factor that mediates signaling between GHR and the nucleus.

GH Activates Akt and Inhibits GSK-3—A link between PI3K and regulation of C/EBPβ by GH is strengthened by the additional observations that GH stimulates the downstream PI3K target Akt and that GH induces phosphorylation and inhibition of GSK-3. Many growth factors have been shown to activate Akt (48). Here GH stimulates Akt phosphorylation on Ser-473. Phosphorylation of Ser-473 and Thr-308 are required for the full activation of Akt (49). It is likely that an enzyme such as PDK1/PDK2 mediates activation of Akt in response to GH, as reported for insulin (49). Autophosphorylation could also contribute to Akt activation, as reported for IGF-1(50). The activation of Akt results in phosphorylation and inhibition of GSK-3 (43). This study shows that GH causes a partial inhibition of GSK-3 most likely by promoting GSK-3 phosphorylation, as shown for GSK-3α. PDGF produced a decrease in GSK-3 activity similar to that produced by GH in the same experiment; the inhibition was comparable to that reported for PDGF in L6 myotubes, nerve growth factor in PC12 cells, and insulin in 3T3-L1 fibroblasts (39, 40, 42, 51). Dependence of GSK-3 phosphorylation on GH-stimulated PI3K is supported by inhibition of GH-promoted GSK-3 phosphorylation by wortmannin. Wortmannin also increased basal GSK-3 activity,[2] suggesting that PI3K may contribute to a tonic restraint of GSK-3 in resting cells. Furthermore, GH was unable to reduce GSK-3 activity below basal levels in wortmannin-treated cells, reinforcing the idea that inhibition of GSK-3 by GH is mediated by PI3K.

C/EBPβ Is a GSK-3 Substrate—GSK-3 substrates include bZIP transcription factors, including C/EBPα, CREB, and c-Jun, as well other transcription factors such as c-Myc (52–54). C/EBPβ contains a putative consensus sequence site for GSK-3 phosphorylation at Ser-184, adjacent to a MAPK site at Thr-188, that is shared by LAP and LIP. In the present studies, both LAP and LIP were phosphorylated by GSK-3 *in vitro*. One can speculate that the GH-mediated inhibition of GSK-3 activity contributes to the dephosphorylation of C/EBPβ. Lithium, which can inhibit GSK3 activity, favored dephosphorylation of LAP and LIP in the presence of GH, supporting a role for GSK-3 in regulation of C/EBPβ phosphorylation by GH. Similarly, insulin stimulates dephosphorylation of C/EBPα through inhibition of GSK-3 in 3T3-L1 adipocytes (52). Insulin and PDGF promote GSK-3 inhibition in 3T3-L1 fibroblasts, resulting in activation of glycogen synthase (42). However, in 3T3-L1 adipocytes, protein phosphatase-1 activation rather than GSK-3 inactivation appears to be the major mechanism by which insulin mediates glycogen synthase dephosphorylation and activation (42). It will be of great interest to determine whether GH can also activate a phosphatase that contributes to C/EBPβ dephosphorylation in 3T3-F442A cells.

Dephosphorylation of C/EBPβ Results in Functionally Important and Distinct Changes in Binding of LAP and LIP to DNA—Regulation of the phosphorylation state of transcription factors is an important mechanism for regulation of gene expression (20). The dephosphorylation of LAP leads to a dramatic increase in its ability to bind to C/EBP sites in the c-*fos* and aP2 promoters. Binding of LAP/LAP homodimers and LAP/LIP heterodimers increased with dephosphorylation of both endogenous and overexpressed LAP. The increased binding associated with dephosphorylation corresponded with the increased LAP binding induced by GH treatment, suggesting that GH-promoted dephosphorylation enhances LAP binding. C/EBPβ contains multiple phosphorylation sites, including sites for Ras-MAPK (Thr-235), calcium/calmodulin-dependent protein kinase (Ser-276), protein kinase C (Ser-105), and protein kinase A (Ser-105, Ser-173, Ser-233, Ser-299) (21, 24, 55–57). However, several reports indicate that phosphorylation of C/EBPβ by PKA and/or PKC attenuates site-selective DNA binding (57), whereas others suggest that phosphorylation may increase binding (58). It remains to be determined which site(s) in LAP are dephosphorylated by GH treatment to increase DNA binding capacity of LAP.

In contrast to the increase in DNA binding that accompanies LAP dephosphorylation, the dephosphorylation of LIP leads to a decrease in its binding to the c-*fos* promoter. This supports the possibility that the reciprocal changes in binding of LAP and LIP with dephosphorylation lead directly to changes in transcription, because LAP can increase and LIP can inhibit transcription mediated by the c-*fos* promoter (14). The differences in DNA binding observed between dephosphorylated LAP and LIP suggest that phosphorylation sites that are not shared by LAP and LIP may be determinants of their abilities to bind to c-*fos* and to regulate transcription. It is not yet known which (if any) of these unshared sites is critical. Although dephosphorylation of LIP led to a decrease in its binding to the c-*fos* C/EBP site, LIP binding to the *aP2* C/EBP site was not decreased by dephosphorylation. This suggests that the consequences of dephosphorylation, at least for LIP, may be specific for the gene to which the protein binds.

In addition to the phosphorylation state, other determinants such as participation in nucleoprotein complexes may also modulate the ability of LAP and LIP to bind to c-*fos* and to regulate transcription. Because mutation of the SRF binding site abolished the binding of C/EBP complexes (7) or of LAP and LIP dimers (data not shown) to the c-*fos* promoter**,** the integrity of SRF binding may contribute to the ability of C/EBPβ to bind to c-*fos*. Furthermore, SRF and C/EBPβ have been shown to interact *in vivo* through the DNA binding domain of SRF and the C terminus of C/EBPβ (59). LAP may thus be recruited to the c-*fos* promoter not only by binding to DNA, but also by protein·protein interactions with SRF, which are reported to be stimulated by activated Ras (59). Mutation of the C/EBP site abolished the binding of LAP and LIP dimers to the c-*fos* promoter, suggesting that they do not bind to the SRE. Interestingly, mutation of the C/EBP site in the c-*fos* upstream regulatory region enhanced the ability of GH to stimulate c-*fos* expression in 3T3-F442A cells stably expressing SRE luc (14), raising the possibility that proteins bound to the C/EBP site might restrain the ability of GH to stimulate c-*fos* promoter activity. It remains to be determined whether interaction(s) of C/EBPβ with other protein(s) bound to the c-*fos* promoter,

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[2] G. Piwien-Pilipuk and J. Schwartz, unpublished.

and/or the recruitment of other factors to an enhanceosome on c-*fos* may participate in the GH-mediated regulation of c-*fos* expression.

Regulation of C/EBPβ by GH—Phosphorylation of LAP and LIP, secondary to overexpression of constitutively active GSK-3, reduces the binding of LAP and LIP. Similarly for c-Jun, gel shift assays using nuclear extracts of cells overexpressing GSK-3 show decreased binding of c-Jun to AP1-sites. c-Jun is phosphorylated by GSK-3 on residues clustered near the DNA binding domain (54). Such results with c-Jun and GSK-3 are similar to those observed here for LAP and LIP binding to the c-*fos* C/EBP site when GSK-3 is co-expressed with LAP or LIP. The proximity of the consensus sequence for GSK-3 phosphorylation of LAP and LIP to the DNA binding domain raises the possibility that, as suggested for c-Jun, charge repulsion via the phosphate groups on amino acids close to the DNA binding domain may interfere with the interaction of this region of the protein with DNA, thereby decreasing LAP and LIP binding in the presence of GSK-3. Future mapping of the phosphorylation sites of GSK-3 in C/EBPβ and mutation of the site(s) of phosphorylation will provide insight into the mechanism of GH-mediated regulation of C/EBPβ phosphorylation and function.

Inhibition of GSK-3 may be critical for GH-stimulated c-*fos* promoter activity, because expression of constitutively active GSK-3 S9A interfered with GH-stimulated transcription via the c-*fos* promoter in the absence and presence of LAP. It has been demonstrated that GSK-3-mediated phosphorylation of c-Jun not only inhibits c-Jun binding to AP-1 sites but also expression of AP-1-sensitive reporter constructs (54, 60–61). It is possible that GSK-3 can modulate binding of complexes to the AP-1 site as well as to the C/EBP site in c-*fos*, thereby reducing GH-stimulated transcription. The decrease in binding of LAP that accompanies its phosphorylation by GSK-3 may therefore be functionally important for the ability of LAP to activate transcription.

In summary, these studies implicate a PI3K pathway in mediating signaling between the GH receptor and the nucleus. GH stimulates PI3K and Akt and inhibits GSK-3, which is implicated in regulating the phosphorylation of C/EBPβ. Changes in the phosphorylation state of LAP and LIP are functionally important in modulating their ability to bind DNA and regulate transcription. Further studies on C/EBPβ phosphorylation will provide more insight into the complex mechanisms by which GH regulates gene expression.

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