THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 46, Issue of November 15, pp. 44557–44565, 2002
Printed in U.S.A.

Dual Regulation of Phosphorylation and Dephosphorylation of C/EBPβ Modulate Its Transcriptional Activation and DNA Binding in Response to Growth Hormone*

Received for publication, July 10, 2002, and in revised form, August 23, 2002
Published, JBC Papers in Press, September 3, 2002, DOI 10.1074/jbc.M206886200

Graciela Piwien-Pilipuk, Ormond MacDougald, and Jessica Schwartz‡

From the Department of Physiology, University of Michigan, Ann Arbor, Michigan 48109

The phosphorylation state of transcription factors is a critical determinant of their function. C/EBPβ occurs in cells as the transcriptional activator liver-enriched activating protein (LAP) and in the truncated form liver-enriched inhibitory protein (LIP) that inhibits transcription. Analysis of C/EBPβ phosphorylation by isoelectric focusing (IEF) shows that LAP is present in multiple forms, each with a different degree of phosphorylation in 3T3-F442A fibroblasts. Growth hormone (GH) treatment induces a new band near the negative pole, consistent with GH-promoted dephosphorylation of LAP. In addition, bands near the positive pole are rapidly and transiently induced, suggesting that GH also stimulates phosphorylation at some site(s) on LAP. C/EBPβ contains a highly conserved MAPK consensus site that corresponds to Thr^{188} in murine (m) LAP and Thr^{37} in mLIP. Immunoblotting with antiphosphopeptide antibodies specific for Thr$^{188/37}$ of C/EBPβ (anti-P-C/EBPβ) shows that GH rapidly and transiently promotes phosphorylation of mLAP and mLIP on the MAPK site. MEK inhibitors prevent this GH-promoted phosphorylation of LAP and LIP, suggesting that such phosphorylation depends on GH-activated MAPK signaling. Mutation of Thr^{235} to Ala in the homologous MAPK site of human (h) LAP (hLAPT235A) inhibits transcription mediated by the c-*fos* promoter in response to GH, indicating that phosphorylation at the MAPK site is required for LAP to be transcriptionally active in the context of GH-stimulated activation of the c-*fos* promoter. Complexes bound to the c-*fos* C/EBP site transiently contain C/EBPβ phosphorylated at the MAPK site. As phosphorylation subsides, the binding of less transcriptionally active forms of LAP increases, consistent with the transient nature of c-*fos* stimulation by GH and other growth factors. Thus, both phosphorylation and dephosphorylation of C/EBPβ, in response to a single physiological stimulus such as GH, coordinately modulate the ability of C/EBPβ to activate transcription by modulating its DNA binding activity and its transactivation capacity.

Phosphorylation of transcription factors is a critical mode of regulation of gene expression (1). Phosphorylation can modulate transcription factor activity by regulating subcellular localization (*e.g.* signal transducers and activators of transcription) (2, 3), modulating DNA binding activity (*e.g.* c-Jun, CCAAT/enhancer-binding protein β (C/EBPβ)[1] (4, 5), modifying protein-protein interactions (*e.g.* cAMP-response element-binding protein (6), or altering transactivation capacity (*e.g.* C/EBPβ, Elk-1 (7–12). These possibilities are not mutually exclusive, and phosphorylation at multiple sites by different kinases, or dephosphorylation by activation of phosphatases or inactivation of kinases can result in dynamic regulation of transcription factors at multiple levels. The integration of multiple changes in phosphorylation is reflected in the functional activity of that transcription factor.

C/EBPβ, a member of the bZIP family of transcription factors, is present in cells as three alternate translation products: 35- and 32-kDa proteins in murine cells known as LAP (liver-enriched activating protein) and a 20-kDa protein known as LIP (liver-enriched inhibitory protein) (13) (Fig. 2*A*). The N-terminal region of LAP corresponds to the transactivation domain, whereas LIP lacks this transactivation domain and acts as an inhibitor of transcription (13). C/EBPβ plays an important role during differentiation of a number of cell types, including adipocytes (14–16), hepatocytes (17), the hematopoietic system (18, 19), and mammary gland (20), as well as in ovulation (21, 22). C/EBPβ knockout mice have alterations in glucose homeostasis (23), show defects in macrophage-dependent antibacterial and antitumor defenses (19), develop lymphoproliferative disorders (24, 25), and have female infertility (22). The participation of C/EBPβ in such a wide variety of physiological events testifies to its versatility, and suggests that the multiple features of its regulation contribute to mediating varied biological outcomes.

C/EBPβ contains phosphorylation sites for multiple protein kinases, including Ras-MAPK for human (h)LAP at Thr^{235} (7), ERK-2 on Thr^{188} of rat (r) LAP (26), glycogen-synthase kinase 3 (GSK-3) at a putative site on Ser^{184} of murine (m)LAP and Ser^{33} of mLIP (5), calcium/calmodulin-dependent protein kinase on Ser^{276} of C/EBPβ (27), protein kinase C (PKC) on Ser^{105} and Ser^{240}, protein kinase A (PKA) on Ser^{105}, Ser^{299}, Ser^{240} of LAP (nuclear factor-interleukin 6) (8, 28, 29), and p90 ribosomal S kinase (p90^{rsk}) on Ser^{105} of rat C/EBPβ and Thr^{217} of murine LAP (30). Thus, differential phosphorylation of C/EBPβ may account for its participation in a wide variety of biological effects. *In vitro* phosphorylation of rat C/EBPβ by

* This work was supported by National Institutes of Health Grant DK46072. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Physiology, University of Michigan Medical School, Ann Arbor, MI 48109-0622. Tel.: 734-647-2124; Fax: 734-647-9523; E-mail: jeschwar@umich.edu.

[1] The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein β; LIP, liver-enriched inhibitory protein; LAP, liver-enriched activating protein; GSK-2, glycogen synthase kinase 3; PKC, protein kinase C; GH, growth hormone; IEF, isoelectric focusing; MAPK, mitogen-activated protein kinase; CHO, Chinese hamster ovary; GHR, growth hormone receptor; CMV, cytomegalovirus; mLAP, murine liver-enriched activating protein; RSV, Rous sarcoma virus; ERK, extracellular signal-regulated kinase; SRE, serum response element.

PKA and/or PKC on Ser^{240}, located in the DNA binding domain, is reported to attenuate site-selective DNA binding (28). GSK-3-mediated phosphorylation of C/EBPβ also decreases DNA binding (5). Phosphorylation on Ser^{105} of rLAP or Thr^{235} of hLAP is also reported to be a key determinant of its transactivation capacity (7, 8, 30). It has been postulated that C/EBPβ contains negative regulatory regions, and that phosphorylation(s) within these domains may regulate C/EBPβ function. Thus, C/EBPβ may be present in cells as a repressed transcription factor that becomes activated upon phosphorylation (31, 32).

Regulation of C/EBPβ by growth hormone (GH) (5, 33, 34) contributes to GH-mediated regulation of transcription of the proto-oncogene c-*fos* (34), which participates in cellular growth pathways (35). Furthermore, GH has been found to promote dephosphorylation of mLAP and mLIP (34). The GH-induced dephosphorylation of C/EBPβ is mediated, at least in part, by GH-stimulated phosphatidylinositol 3-kinase/Akt signaling, resulting in inhibition of GSK-3 (5). Dephosphorylation of LAP leads to an increase in binding of LAP-containing complexes to C/EBP sites in the *c-fos* and *aP2* promoters. Conversely, GSK-3-induced phosphorylation of LAP decreases binding to the c-*fos* C/EBP site (5). Furthermore, GH-promoted inhibition of GSK-3 is required for GH to stimulate c-*fos* promoter activity.

The present study examines which among the multiple phosphorylation site(s) of LAP and LIP are functionally important for transcriptional activation, in the context of GH-stimulated c-*fos* promoter activation. By isoelectric focusing (IEF) LAP was found to be present in 3T3-F442A cells in several forms exhibiting different degrees of phosphorylation. IEF revealed that GH promotes not only dephosphorylation, but also a rapid and transient phosphorylation of murine C/EBPβ on Thr^{188} in mLAP and Thr^{37} in mLIP, sites that correspond to the same conserved mitogen-activated protein kinase (MAPK) consensus sequence in each form. Mutational analysis of hLAP at Thr^{235} (homologous to Thr^{188} in mLAP) indicates that this phosphorylation is critically important for LAP to be transcriptionally active in the context of the GH-stimulated c-*fos* promoter. Changes in DNA binding associated with changing levels of phosphorylated and dephosphorylated C/EBPβ may contribute to the transient pattern of GH-stimulated c-*fos* expression. Thus, C/EBPβ is a highly phosphorylated protein whose function is dynamically modulated by both phosphorylation and dephosphorylation in response to a single physiological regulator.

EXPERIMENTAL PROCEDURES

Materials—Murine 3T3-F442A preadipocyte fibroblasts, which are highly responsive to GH through endogenous GH receptors (36), 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) (37). In CHO-GHR cells GH induces c-*fos* mRNA and stimulates transcriptional activation via the SRE-C/EBP site to the same extent as in CHO cells expressing full-length GHR (38). Human embryonic kidney 293T cells were provided by Dr. M. Lazar (University of Pennsylvania). Recombinant human GH was provided by Eli Lilly, Inc. Culture media, calf serum, fetal calf serum, G418, L-glutamine, and antibiotic-antimycotic were purchased from Invitrogen. Bovine serum albumin (CRG7) was purchased from Intergen. The MEK inhibitor U0126 and luciferin were purchased from Promega. PD098059 was purchased from Sigma, and alkaline phosphatase and Complete™ protease inhibitor mixture (EDTA-free) were purchased from Roche Molecular Biochemicals. Bradford reagent (Sigma) was used to determine the concentration of protein in cell or nuclear extracts for all experiments. [α-^{32}P]dATP was purchased from PerkinElmer Life Sciences. β-Galactosidase chemiluminescence reagent was purchased from Tropix. The ECL detection system was purchased from Amersham Biosciences.

Cell Culture and Hormone Treatment—3T3-F442A preadipocyte fibroblasts and 293T human embryonic kidney 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 μg/ml streptomycin, and 0.25 μg/ml amphotericin. Prior to treatment, cells were deprived of serum by incubation overnight in the appropriate medium containing 1% bovine serum albumin instead of serum. Then cells were incubated with or without GH at 500 ng/ml (23 nM) or as indicated in figure legends.

Plasmids and Antibodies—A plasmid encoding mLAP driven by the CMV promoter (CMV-mLAP) was kindly provided by U. Schibler (University of Geneva) and L. Sealy (Vanderbilt University). Plasmids encoding hLAP (also known as NF-IL-6) and a mutant hLAP where Thr^{235} was mutated to Ala (hLAP-T235A) were kindly provided by Dr. S. Akira (Osaka University, Japan) (7) courtesy of L. Sealy. The plasmid *fos*-Luc, provided by Dr. W. Wharton (University of S. Florida) contains 379 bp of the mouse c-*fos* promoter immediately 5′ of the transcription start site, cloned upstream of the luciferase gene (39). The plasmid RSV-β-galactosidase was provided by Dr. M. Uhler (University of Michigan). pcDNA3.1 was purchased from Clontech.

Specific rabbit polyclonal antibodies against a synthetic phospho-Thr^{235} peptide (keyhole limpecthemocyanin coupled) corresponding to residues surrounding Thr^{235} of human C/EBPβ (identical to Thr^{188} in mC/EBPβ) (anti-P-C/EBPβ) were provided by Cell Signaling Technology, Inc. (Beverly, MA). Specific rabbit polyclonal antibodies against a peptide corresponding to amino acids 278–295 at the C terminus of C/EBPβ (anti-C/EBPβ) were purchased from Santa Cruz Biotechnology, Inc. Antibodies against phosphorylated extracellular signal-regulated kinases (ERK)1/ERK2 (anti-P-ERK1/2) or antibodies against total ERK1/ERK2 (anti-ERK1/2) were purchased from Promega.

IEF—Nuclei from 3T3-F442A fibroblasts were prepared as previously described (34). Nuclei were lysed in urea buffer (9 M urea, 1% Nonidet P-40, 1% dithiothreitol). Nuclear lysate was sonicated and centrifuged at 13,500 rpm at 4 °C for 15 min, and the supernatant was stored at −80 °C. For alkaline phosphatase treatment, nuclei, prepared as described (34), were incubated in the presence or absence of 200 units of alkaline phosphatase for 1 h at 37 °C; the reaction was stopped by adding an equivalent volume of 9 M urea buffer. Polyacrylamide-urea minigels were made according to the manufacturer's instructions (Bio-Rad) with a 1:4:1 mixture of pH 3 to 10, pH 5 to 8, and pH 8 to 10.5 ampholytes, respectively. Protein samples (15–20 μg) were loaded and focused for 15 min at 100 V, 15 min at 200 V, and 1.5 h at 450 V. Proteins were then transferred to Immobilon™ membrane for immunoblot analysis using anti-C/EBPβ (1:1000) or anti-P-C/EBPβ (1:500).

Immunoblotting—Whole cell lysates from 3T3-F442A fibroblasts were prepared and analyzed (35–40 μg) by immunoblotting as previously described (34) using anti-P-C/EBPβ (1:500) or anti-C/EBPβ (1:1000). In some experiments 3T3-F442A cells were preincubated 30 min with the indicated concentrations of the MEK inhibitors U0126 or PD098059 prior to addition of GH (5 min). Membranes were stripped and reprobed with anti-P-ERK 1/2 (1:5000) or anti-ERK 1/2 (1:5000), as previously described (12). The apparent M_r are based on prestained molecular weight standards (Invitrogen).

Electrophoretic Mobility Shift Assay (EMSA)—Cell extracts enriched in mLAP, hLAP, or hLAP-T235A were obtained by transfection of 293T cells with 1 μg of CMV-mLAP, CMV-hLAP, or CMV-hLAP-T235A DNA, as previously described (5). Nuclear extracts from 3T3-F442A cells were prepared as previously described (34), and a volume of nuclear extracts containing 8–10 μg of protein was used for EMSA. Binding reactions proceeded for 30 min at room temperature with ^{32}P-labeled oligonucleotide containing the c-*fos* C/EBP site and flanking SRE (C/EBP-SRE) (34) or the C/EBP site of the *aP2* promoter (34, 40) as described (5). In some experiments, cell extracts were incubated for 20 min at room temperature with 1 μl of anti-CEBPβ (1:10 final dilution), anti-P-CEBPβ (1:10 final dilution), or nonimmune (1:10 final dilution) antiserum prior to EMSA, as indicated in the figure legends. Complexes were separated by nondenaturing 7% PAGE followed by autoradiography.

Gene Expression Assay—CHO-GHR cells (1×10^5 cells/35 mm well) were transiently transfected by calcium phosphate coprecipitation (41) with c-*fos*-Luc (0.4 μg) and RSV-β-galactosidase (0.1 μg) plasmids, in the presence or absence of CMV-hLAP (2 ng), CMV-hLAPT235A (2 ng), or corresponding amounts of pcDNA3.1 vector per well. Twenty-four h after transfection, cells were deprived of serum by incubation in medium containing 1% bovine serum albumin for 18 h prior to treatment.

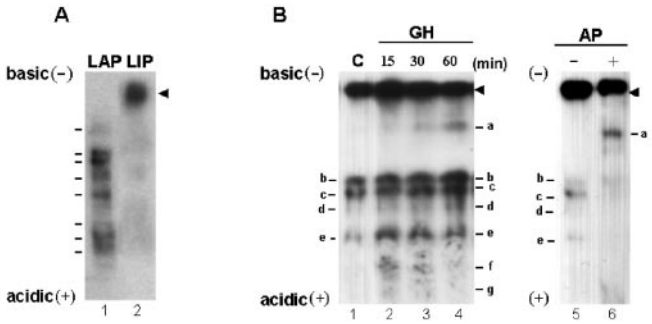

FIG. 1. **Multiple phosphorylated forms of endogenous C/EBPβ are regulated by GH.** *A*, extracts from 293T cells overexpressing mLAP (*lane 1*) or mLIP (*lane 2*) were resolved by IEF and subjected to immunoblot analysis with anti-C/EBPβ (1:1000). Basic (−) and acidic (+) ends of the blot are indicated. LAP (isoelectric point = 8.4) is resolved into 8 bands when samples are focused to equilibrium with ampholyte mixture (see "Experimental Procedures"). LIP (isoelectric point = 10.42) is not resolved with the ampholyte mixture (*arrowhead*). Similar results were obtained in three independent experiments. *B*, 3T3-F442A cells were treated with vehicle (*lane 1*) or GH (*lanes 2–4*) for the indicated periods of time. Nuclear proteins were prepared, separated by IEF, and subjected to immunoblot analysis for C/EBPβ. Nuclear extracts from 3T3-F442A cells were incubated without (*lane 5*) or with alkaline phosphatase (*AP*) (*lane 6*) prior to IEF. The cathodic (basic, −) and the anodic (acidic, +) migration of the proteins is indicated. Similar results were obtained in four independent experiments.

Cell lysates were prepared as described previously (34), 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 in each experiment. A two-sample *t* test was used (SigmaStat) to judge statistical significance. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Phosphorylation as Well as Dephosphorylation of C/EBPβ Is Induced by GH—GH promotes a rapid and transient dephosphorylation of endogenous mLAP and mLIP in 3T3-F442A preadipocytes (34). Similar promotion of LAP and LIP dephosphorylation was also induced by insulin-like growth factor-1 and insulin.[2] However, in all cases, a considerable amount of phosphorylated C/EBPβ is still evident on immunoblots.

Because both LAP and LIP contain phosphorylation sites for multiple kinases, C/EBPβ may be present as multiple phosphorylated species not resolved by conventional SDS-PAGE. IEF was used to analyze C/EBPβ, because IEF separates proteins according to charge density in a pH gradient generated in the gel. mLAP overexpressed in 293T cells is resolved into at least 8 bands by IEF (Fig. 1*A*, *lane 1*). This suggests that overexpressed mLAP is present in multiple forms with different degrees of phosphorylation. As expected, overexpressed mLIP is detected as a single band that runs close to the negative pole (Fig. 1*A*, *lane 2*), consistent with LIP being a basic protein (isoelectric point = 10.42), which is not further resolved under the conditions of IEF used.

Endogenous LAP from untreated 3T3-F442A fibroblasts can be resolved into at least 4 bands (Fig. 1*B*, *lane 1, bands b-e*), indicating that endogenous mLAP is present in quiescent cells in multiple forms with different degrees of phosphorylation. The dense uppermost band at the basic end (Fig. 1*B*, *arrowhead*) in this and subsequent figures is thought to correspond to the migration of endogenous LIP because it coincides with migration of overexpressed mLIP.

The pattern of migration of mLAP on IEF changes in several ways upon treatment of the cells with GH. Some bands that migrate closer to the acidic end (positive pole) increase in intensity over 15–60 min (Fig. 1*B*, *lanes 2–4, bands b-e*). Other bands that migrate toward the acidic end are induced (*bands f* and *g*) by 15–30 min after GH treatment. These more acidic bands may correspond to more phosphorylated forms of LAP, because upon alkaline phosphatase treatment of control 3T3-F442A nuclear extracts bands *b, c, d,* and *e* are reduced in intensity, shifting to band *a* (Fig. 1*B*, *lane 6 versus 5*). The new band *a* close to the basic end (Fig. 1*B*, *lanes 3–4, band a*) appears after 30–60 min of GH treatment. The GH-induced band *a* is thought to correspond to a less phosphorylated or dephosphorylated form of LAP. In support of this, band *a* comigrates with the band that appears upon alkaline phosphatase treatment of control extracts (Fig. 1*B*, *lane 6, band a*), suggesting that band *a* corresponds to a dephosphorylated form of mLAP. Thus, IEF reveals multiple phosphorylated forms of mLAP, and suggests that both its phosphorylation (15–60 min) and dephosphorylation (30–60 min) are modulated by GH.

Phosphorylation of Murine C/EBPβ at a MAPK Consensus Site Is Rapidly Stimulated by GH—C/EBPβ contains a highly conserved MAPK consensus site that corresponds to Thr^{188} in mLAP and Thr^{37} in mLIP (Fig. 2*A*). Because IEF suggests that GH promotes phosphorylation of LAP, and GH is known to stimulate the MAPKs ERKs 1 and 2 (42–45), the effect of GH on the phosphorylation of the MAPK site in C/EBPβ was investigated. An antibody made against a peptide that encompasses the phosphorylated Thr in the conserved MAPK consensus sequence in C/EBPβ (anti-P-C/EBPβ) reveals the rapid and transient appearance of bands that migrate at the same positions as mLAP and mLIP upon GH treatment of 3T3-F442A fibroblasts (Fig. 2*B*, *upper panels, lanes 2* and *3*). The bands are evident within 5 min of GH (Fig. 2*B*, *lane 2*), and subside in 30–60 min (*lanes 4–7*). The *lower panels* show total mLAP and mLIP present in each condition. These data suggest that GH promotes a rapid and transient phosphorylation of mLAP on Thr^{188} and of mLIP on Thr^{37}. Similar stimulation of LAP and LIP phosphorylation was also observed after 5 min treatment of 3T3-F442A cells with insulin-like growth factor-1, platelet-derived growth factor, LIF, or serum, but not with interferon γ, as detected with anti-P-C/EBPβ (data not shown).

IEF was used in conjunction with anti-P-C/EBPβ to examine which of the multiple phosphorylated forms of mLAP is phosphorylated on Thr^{188} in response to GH. Immunoblot analysis with anti-P-C/EBPβ of the nuclear proteins separated by IEF shows that the antibody recognizes multiple bands that appear within 5 min of GH treatment of 3T3-F442A cells (Fig. 2*C*, *lane 2, bands d-j*); these bands subside and are no longer detected 30–60 min after GH treatment (Fig. 2*C*, *lanes 3* and *4*). Because multiple bands are detected by anti-P-C/EBPβ, different forms of mLAP appeared to be phosphorylated at Thr^{188} in response to GH. Anti-P-C/EBPβ also recognizes a band close to the basic end that appears within 5 min of GH and subsides in 30–60 min (Fig. 2*C*, *lane 2* and *3, arrowhead*); this very basic band is thought to correspond to LIP phosphorylated on Thr^{37}. All bands that correspond to mLAP or mLIP are detected when the membrane is reprobed with anti-C/EBPβ (Fig. 2*C*, *right panel*).

Increasingly darker bands representing phosphorylated LAP and LIP on immunoblots appear upon treatment of 3T3-F442A cells with increasing concentrations of GH (Fig 3, *upper panel, lane 3 versus 1*). Phosphorylation of LAP and LIP in response to GH (5 min) is evident with concentrations of GH as low as 5 ng/ml (*lane 3*) and increases to an apparent maximum at 100 ng/ml GH (*lanes 6–8*). These data are consistent with GH rapidly promoting the phosphorylation of mLAP on Thr^{188} and mLIP on Thr^{37}. Furthermore, phosphorylation and activation of ERK 1/2, detected by immunoblotting with anti-phospho-

[2] A. Sieh, A. Sachdeva, and J. Schwartz, unpublished data.

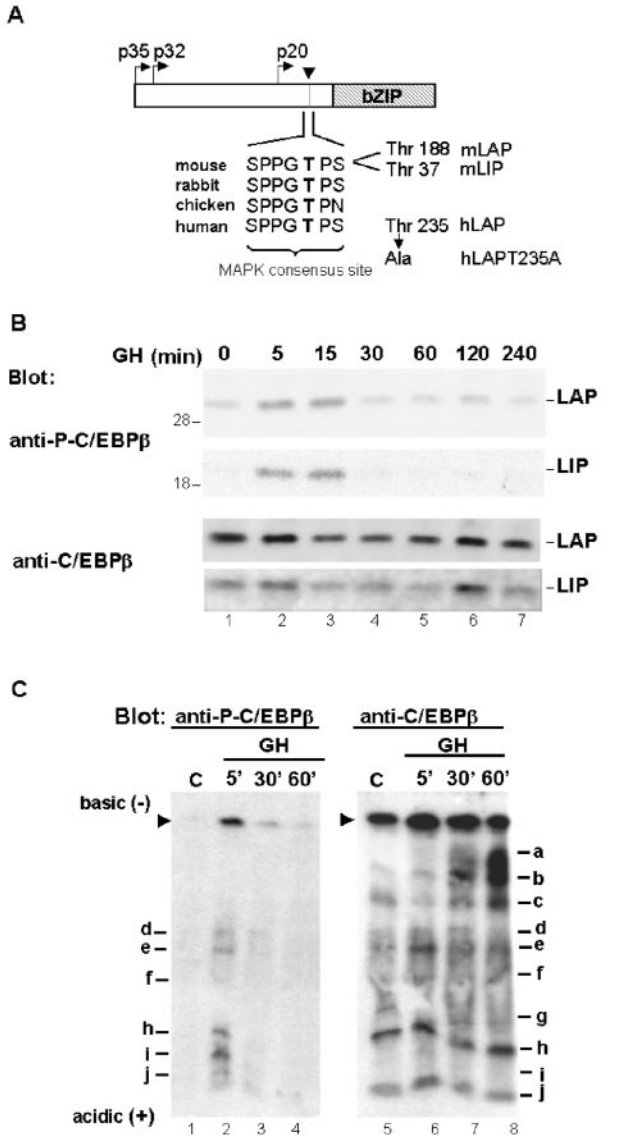

FIG. 2. **GH promotes a rapid and transient phosphorylation of LAP and LIP.** *A*, schematic representation of C/EBPβ showing start sites (*bent arrows*) for three alternate translation products, the sequence of the conserved MAPK consensus site (*arrowhead*) in different species, and the mutation site of Thr^{235} to Ala in hLAP. *B*, 3T3-F442A fibroblasts were incubated with vehicle (*lane 1*) or GH (500 ng/ml) for 5–240 min as indicated (*lanes 2*-7). The cells were lysed and used for immunoblotting with antibodies against a C/EBPβ peptide phosphorylated at the position corresponding to Thr^{188} of mLAP (anti-P-C/EBPβ, 1:500). Bands representing LAP and LIP are shown. $M_r \times 10^{-3}$ is designated on the *left*. Blots were stripped and reprobed with anti-C/EBPβ (1:1000) to determine total C/EBPβ (*lower panels*). Similar findings were obtained in three independent experiments. *C*, 3T3-F442A cells were treated as in *part A*, nuclear proteins were separated by IEF and subjected to immunoblot analysis with anti-P-C/EBPβ (1:500, *lanes 1–4*) or anti-C/EBPβ (1:1000, *lanes 5–8*). Acidic (+) and basic (−) ends of the blot are indicated. Similar results were obtained in two independent experiments.

ERK1/2, parallels phosphorylation of LAP and LIP in a GH dose-dependent manner (Fig. 3, *lower panels, lanes 3–8*). Taken together these results suggest that GH promotes a rapid and transient phosphorylation of LAP and LIP at the conserved MAPK site, possibly through activation of ERKs1/2.

Phosphorylation of LAP and LIP Depends on MAPK Activation—To investigate whether GH-regulated phosphorylation of LAP and LIP depends on GH-stimulated MAPK activity, the effect of pharmacological agents that block the MAPK cascade

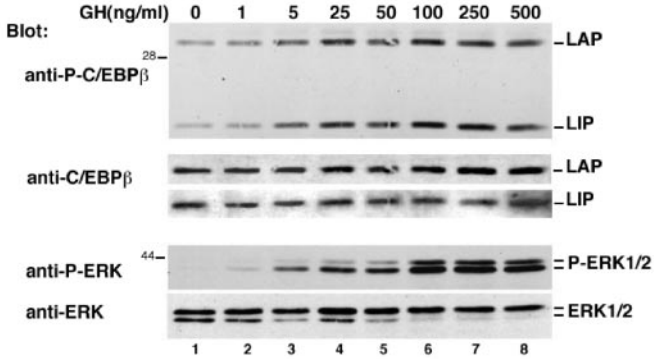

FIG. 3. **LAP and LIP phosphorylation corresponds with ERK 1/2 activation upon GH treatment.** 3T3-F442A fibroblasts were incubated with vehicle (*lane 1*) or increasing concentrations of GH as indicated (*lanes 2–8*) for 5 min. Cell lysates were immunoblotted with anti-P-C/EBPβ (1:500) (*upper panel*) or anti-C/EBPβ (1:1000) (*middle panels*). Blots were stripped and reprobed with anti-P-ERK1/2 or anti-ERK 1/2, respectively (*bottom panels*). $M_r \times 10^{-3}$ are indicated on the *left*. Data are representative of two independent experiments.

was tested on GH-promoted phosphorylation of LAP and LIP. Treatment of cells with increasing concentrations of the MEK inhibitor U0126 (46) results in a dose-dependent reduction of the GH-stimulated bands recognized by anti-P-C/EBPβ (Fig. 4, *upper panel, lanes 4, 6,* and *8 versus 2*). A complete inhibition of the GH-induced bands is achieved with 5–10 μM U0126. Inhibition of the GH-induced bands detected by anti-P-C/EBPβ is also obtained when cells are treated with another MEK inhibitor PD098059 (40 μM) (data not shown). Immunoblotting with anti-C/EBPβ indicates that C/EBPβ is present in each condition whether or not the proteins are phosphorylated at the MAPK site (Fig. 4, *middle panels*). The effectiveness of U0126 in inhibiting the GH stimulated activity of ERKs 1 and 2 is evident when the membrane is reprobed with anti-P-ERK1/2 antibody (Fig. 4, *lower panels*). The inhibition of GH-promoted phosphorylation of ERK 1/2 by U0126 (*lanes 4, 6,* and *8*) parallels the inhibition of GH-promoted phosphorylation of LAP and LIP, whereas total ERKs remain constant (Fig. 4, *lowest panel*). Taken together, these results suggest that ERKs 1/2 contribute to phosphorylation of mLAP and mLIP at Thr^{188} and Thr^{37}, respectively, in GH-treated 3T3-F442A cells.

Phosphorylation of LAP at the MAPK Site Is Necessary for GH-stimulated c-fos Promoter Activation—The MAPK consensus site is highly conserved throughout species, encompassing Thr^{188} in murine LAP and the homologous Thr^{235} in human (h)LAP (Fig. 2*A*). To examine whether GH-promoted phosphorylation of LAP at its MAPK consensus site is required for LAP to be transcriptionally active, hLAP or mutant hLAPT235A, where Thr^{235} was mutated to Ala (Fig. 2*A*), was expressed in combination with a luciferase reporter gene driven by the c-*fos* promoter (*fos*-Luc) in GH-responsive CHO-GHR cells. Treatment of cells with GH produces its typical doubling of luciferase expression mediated by the c-*fos* promoter (Fig. 5, *left pair of bars*), as reported previously (34). Overexpression of hLAP increases the basal level of luciferase (Fig. 5, *open bars, hLAP versus vector, open bars*), as previously shown for mLAP (34), consistent with LAP being an activator of transcription. GH similarly doubles *c-fos* promoter activity in the presence of hLAP (Fig. 5, *middle pair of bars*); this is similar to previous observations with mLAP (34). In contrast, in the presence of mutant hLAPT235A, GH fails to promote a significant increase in c-*fos* promoter activity (Fig. 5, *right pair of bars*). These findings suggest that phosphorylation of LAP at Thr^{235} in the MAPK consensus site is required for LAP to be transcriptionally active in the context of the c-*fos* promoter in response to GH.

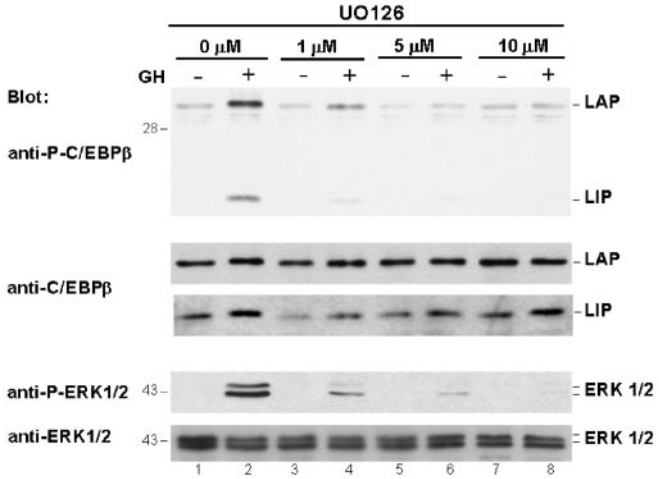

FIG. 4. **Phosphorylation of LAP and LIP at Thr^{235} depends on MAPK activation.** 3T3-F442A cells were treated with vehicle (0.05% Me_2SO) (*lanes 1* and *2*) or with increasing concentrations of U0126, a MAPK inhibitor, for 30 min, as indicated (*lanes 3–8*). Then GH (*lanes 2, 4, 6,* and *8*) or its vehicle (*lanes 1, 3, 5,* and *7*) were added for an additional 5 min. Cell lysates were immunoblotted with anti-P-C/EBPβ (1:500) (*upper panel*) or anti-C/EBPβ (1:1000) (*middle panels*). Blots were stripped and reprobed with anti-P-ERK1/2 (1:5000) and anti-ERK 1/2 (1:5000) (*bottom panels*). $M_r \times 10^{-3}$ are indicated on the *left*. Similar findings were obtained in three independent experiments.

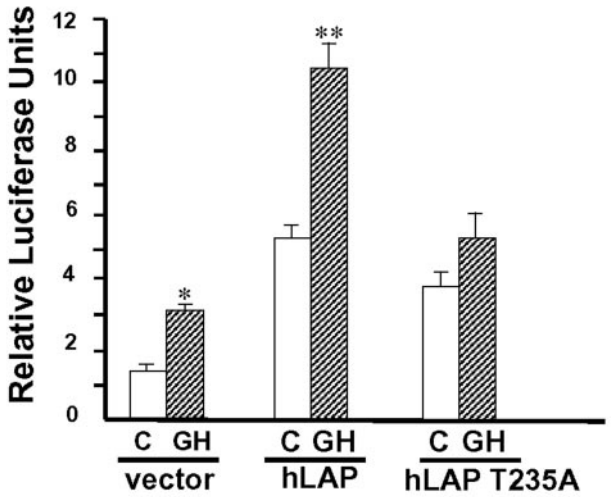

FIG. 5. **Mutation of Thr^{235} in hLAP interferes with GH-stimulated c-*fos* promoter activity.** CHO-GHR cells were transiently transfected with CMV-hLAP (*hLAP*), CMV-hLAPT235A (*hLAP T235A*), or empty vector, along with c-*fos*-luc and RSV-β-galactosidase plasmids. After 24 h of transfection, cells were deprived of serum overnight and 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 five independent experiments. Luciferase activity in response to GH was significantly greater than its respective control in the presence of vector (*, $p < 0.002$) or hLAP (**, $p < 0.05$). There was not a significant difference between control and GH-treated cells in the presence of hLAPT235A ($p > 0.05$).

DNA Binding of LAP Depends on Its Phosphorylation State—We have previously shown that dephosphorylation of mLAP by GH dramatically increases the binding of mLAP dimers to the c-*fos* C/EBP site (5). Because the present studies show that GH also promotes a rapid phosphorylation of LAP (Fig. 2), which is required for LAP to be transcriptionally active (Fig. 5), the effect of phosphorylation of the MAPK consensus site of LAP on its DNA binding activity was examined. Extracts from 293T cells overexpressing mLAP, hLAP, or hLAPT235A were subjected to EMSA to detect LAP complexes bound to the

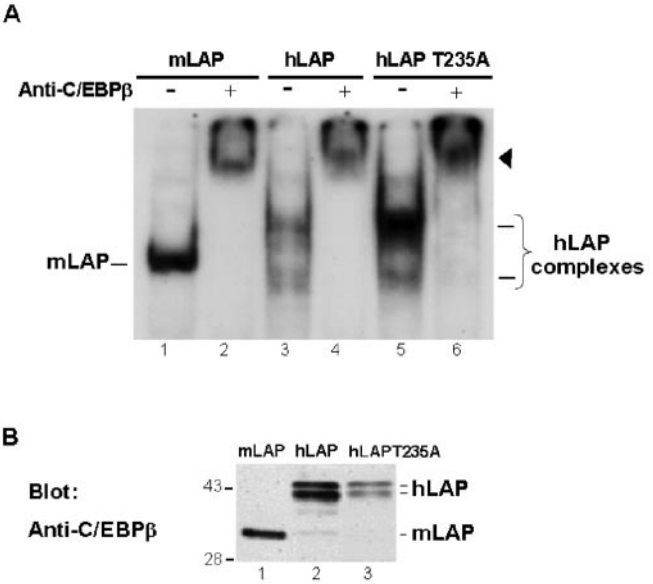

FIG. 6. **hLAP and mutant hLAPT235A bind to the c-*fos* C/EBP site.** *A*, extracts from 293T cells overexpressing mLAP, hLAP, or hLAPT235A were incubated in the absence (*lane 1*, *3*, and *5*) or presence (*lane 2*, *4*, and *6*) of antiserum specific for C/EBPβ. Complexes containing mLAP (*lanes 1* and *2*), hLAP (*lanes 3* and *4*), or hLAPT235A (*lanes 5* and *6*) were identified by EMSA using the C/EBP-SRE probe. The *arrowhead* indicates the supershift with anti-C/EBPβ. *B*, extracts used in *part A* containing mLAP (*lane 1*), hLAP (*lane 2*), or hLAPT235A (*lane 3*) were analyzed by immunoblotting with antibody specific for C/EBPβ. $M_r \times 10^{-3}$ are indicated on the *left*. Data are representative of five independent experiments.

c-*fos* C/EBP site. The binding of mLAP appears as a band (Fig. 6A, *lane 1*) which is supershifted by anti-C/EBPβ (Fig. 6*A*, *lane 2, arrowhead*), as shown previously (5). hLAP was also found to bind to the c-*fos* C/EBP site (Fig. 6*A*, *lane 3*). The migration of hLAP complexes appears as 2–3 bands that migrate more slowly than mLAP, and which appear more diffuse. The bands corresponding to hLAP-DNA complexes are also supershifted in the presence of anti-C/EBPβ (Fig. 6*A*, *lane 4, arrowhead*).

Mutant hLAPT235A, in which the conserved Thr^{235} is mutated to Ala, also binds to the c-*fos* C/EBP site (Fig. 6*A*, *lane 5*), comigrating with wild type hLAP. Furthermore, the hLAPT235A-DNA complexes are supershifted in the presence of anti-C/EBPβ (Fig. 6A, *lane 6 versus 5*, *arrowhead*). The DNA binding of hLAPT235A appears more intense than binding of wild type hLAP (Fig. 6*A*, *lane 5 versus 3*), particularly when the relatively lower expression of hLAPT235A is taken into consideration (Fig. 6*B*, *lane 3 versus 2*). Similar results are obtained when the C/EBP site of the *aP2* gene is used as a probe (data not shown). The increased binding to DNA with mutation of Thr^{235} suggests that less phosphorylated forms of hLAP may bind more efficiently to the c-*fos* C/EBP site. Nevertheless, hLAPT235 was transcriptionally inactive when GH-stimulated c-*fos* promoter activity was measured.

hLAP and hLAPT235A Show Different Patterns of Phosphorylation—Because mLAP shows multiple phosphorylated forms by IEF (Fig. 1*A*), the effect of mutation of Thr^{235} in hLAP on the pattern of phosphorylation of hLAP was examined. Cell extracts enriched in mLAP, hLAP, or hLAPT235A were treated or not with alkaline phosphatase and resolved by SDS-PAGE or IEF. Alkaline phosphatase treatment caused mLAP to shift to a faster mobility form on immunoblots (Fig. 7*A*, *lane 2 versus 1*), consistent with mLAP dephosphorylation, as shown previously (5). However, for hLAP (*lane 4 versus 3*) or hLAPT235A (*lane 6 versus 5*) no change in migration was detectable on immunoblots after alkaline phosphatase treatment. Immuno-

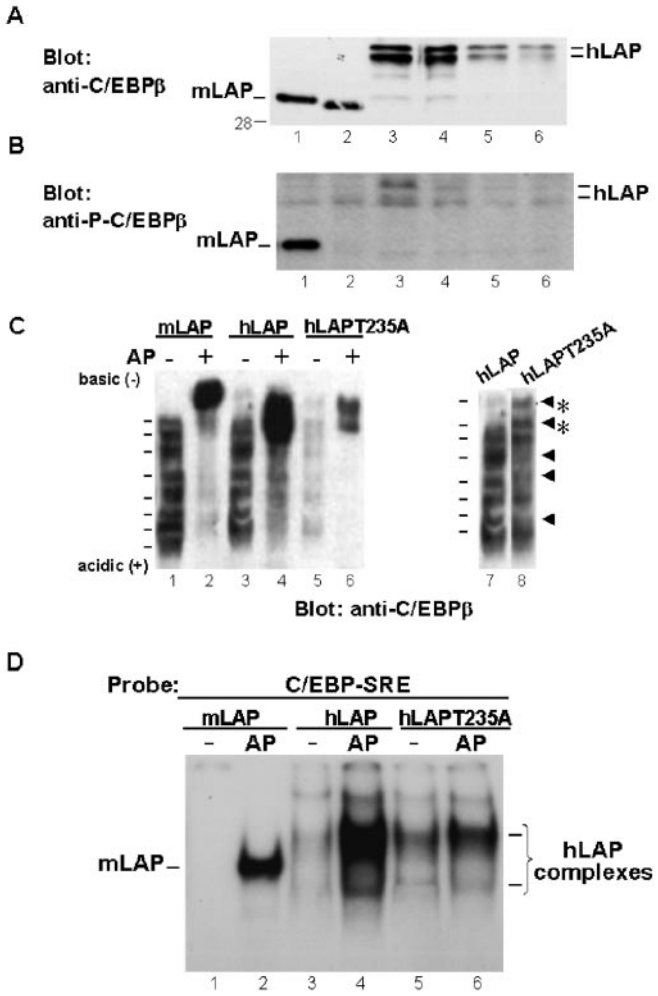

FIG. 7. **The multiple phosphorylated forms of mLAP, hLAP, and hLAPT235A show distinct IEF patterns.** *A*, extracts from 293T cells overexpressing mLAP, hLAP, or hLAPT235A were treated without (*lanes 1*, *3*, and *5*) or with alkaline phosphatase (AP, 200 units) (*lanes 2*, *4*, and *6*) prior to immunoblot analysis with antibody specific for C/EBPβ (1:1000). $M_r \times 10^{-3}$ are indicated on the *left*. *B*, blots were stripped and reprobed with anti-P-C/EBPβ (1:500). *C*, extracts from 293T cells expressing mLAP (*lanes 1* and *2*), hLAP (*lanes 2* and *3*), or hLAPT235A (*lanes 4* and *5*) treated without (*lanes 1*, *3*, *5*, *7*, and *8*) or with alkaline phosphatase (*AP*, *lanes 2*, *4*, and *6*) were resolved by IEF and subjected to immunoblot analysis with anti-C/EBPβ (1:1000). Basic (−) and acidic (+) ends of the blot are indicated. Longer exposure of bands resolved for hLAPT235A in *lane 5* are presented in *lane 8* for comparison with bands resolved for wild type hLAP (*lanes 3* and *7*). The *arrowheads* in the *right margin* indicate bands that are absent or reduced in hLAPT235A and *arrowheads with the asterisk* indicate bands increased in hLAPT235A with respect to wild type hLAP. Similar results were obtained in three independent experiments. *D*, extracts from 293T cells overexpressing mLAP, hLAP, or hLAPT235A were treated without (*lanes 1*, *3*, and *5*) or with alkaline phosphatase (AP, 200 units) (*lanes 2*, *4*, and *6*) prior to incubation with the C/EBP-SRE probe for EMSA. Similar results were obtained in four independent experiments.

blotting of the same extracts with anti-P-C/EBPβ reveals a band that corresponds with the size of mLAP (Fig. 7*B*, *lane 1*) as well as the slower migrating hLAP (Fig. 7*B*, *lane 3*), suggesting that overexpressed mLAP and hLAP are constitutively phosphorylated at the MAPK site. As expected, after lysates are treated with alkaline phosphatase, mLAP and hLAP are undetectable with anti-P-C/EBPβ (Fig. 7*B*, *lane 2 versus* 1, *lane 4 versus 3*). Importantly, anti-P-C/EBPβ was unable to detect mutated hLAPT235A without (Fig. 7*B*, *lane 5*) or with alkaline phosphatase treatment (*lane 6*), indicating the specificity of the antibody in recognizing the phosphorylation on Thr^{235} of hLAP.

Because changes in the phosphorylation state of hLAP or hLAPT235A could not be resolved by conventional SDS-PAGE, IEF was used. mLAP overexpressed in 293T cells is resolved into at least 8 bands by IEF (Fig. 7*C*, *lane 1*; Fig. 1*A*), indicating that the overexpressed mLAP, like endogenous mLAP (Fig. 1*B*, *lane 1*), exhibits forms with heterogeneous degrees of phosphorylation. Alkaline phosphatase treatment of mLAP followed by IEF caused almost complete loss of the most acidic (phosphorylated) forms and the accumulation of more basic (dephosphorylated) forms (Fig. 7*C*, *lane 2*). These results are consistent with the more acidic species of mLAP corresponding with forms containing more phosphate groups, and with the appearance of a more rapidly migrating band detected by SDS-PAGE upon alkaline phosphatase treatment of mLAP (Fig. 7*A*, *lane 2 versus 1*).

hLAP is also resolved into at least 8 bands by IEF (Fig. 7*C*, *lanes 3*) indicating that overexpressed hLAP is also present in forms with different degrees of phosphorylation, with the more acidic species containing more phosphate groups. Alkaline phosphatase treatment of hLAP causes a shift of more acidic to basic forms (Fig. 7*C*, *lane 4 versus 3*), consistent with alkaline phosphatase-dependent dephosphorylation of hLAP. Thus, IEF confirms that alkaline phosphatase causes dephosphorylation of hLAP, even though no change in migration of hLAP had been detectable by SDS-PAGE after alkaline phosphatase treatment (Fig. 7*A*, *lane 3 versus 4*).

hLAPT235A also appears to be resolved as at least 8 bands by IEF (Fig. 7*C*, *lanes 5* and *8*, which is a darker exposure of *lane 5*). Alkaline phosphatase treatment shifts the migration of the more acidic forms of hLAPT235A toward more basic forms (Fig. 7*C*, *lane 6 versus 5*), consistent with the appearance of less phosphorylated or dephosphorylated forms of hLAPT235A. Importantly, direct comparison between hLAP and hLAPT235A (Fig. 7*C*, *lanes 7* and *8*) reveals significant differences in their patterns of migration upon IEF. hLAPT235A presents more basic bands at the top of the gel (*lane 8, arrowheads with asterisks*). Furthermore, at least three of the more acidic bands are decreased (*lane 8, arrowheads without asterisks*). The pattern of hLAPT235A migration indicates that the mutant hLAP is present in several forms with a lower degree of phosphorylation than wild type hLAP. This suggests that mutation of Thr^{235} not only abolishes phosphorylation of that particular residue, but also alters phosphorylation at other site(s).

The lesser degree of phosphorylation on mutant LAP provided a means to evaluate whether less phosphorylated forms of hLAPT235A account for its higher DNA binding activity compared with wild type hLAP. Extracts enriched in mLAP, hLAP, and hLAPT235A were incubated with alkaline phosphatase prior to EMSA. For reference, dephosphorylation of mLAP is shown to cause a dramatic increase in its binding to the c-*fos* C/EBP site (Fig. 7*D*, *lane 2 versus 1*) as shown previously (5). Alkaline phosphatase treatment of hLAP caused a substantial increase in its binding also (Fig. 7*D*, *lane 4 versus 3*). If less phosphorylated hLAPT235A binds more efficiently to DNA, then further dephosphorylation of hLAPT235A might be expected to increase its DNA binding activity even more. Indeed, alkaline phosphatase treatment of hLAPT235A increased the binding of hLAPT235A even more than the mutation alone (Fig. 7*D*, *lane 6 versus 5*), suggesting that less phosphorylated forms of hLAPT235A may bind *in vitro* to DNA more efficiently than more phosphorylated forms. Taken together these results show that overexpressed mLAP, hLAP, and hLAPT235A occur in multiple forms with heterogeneous degrees of phosphorylation, and that hLAPT235A is present in forms with fewer phosphate groups compared with wild type hLAP. The higher DNA binding of hLAPT235A compared with wild type hLAP

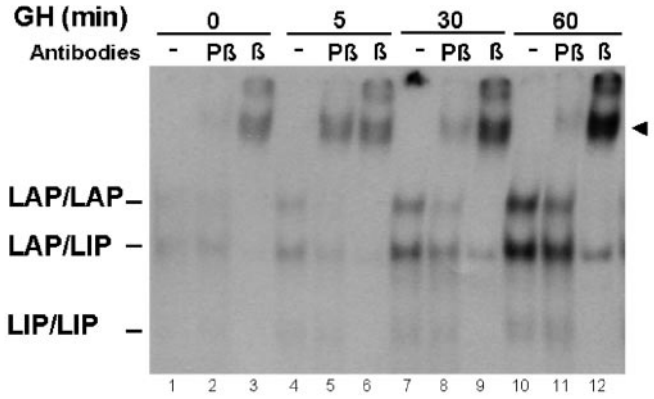

FIG. 8. **Phosphorylated C/EBPβ is present transiently in GH-stimulated complexes bound to DNA.** Nuclear extracts from 3T3-F442A cells treated without (*lanes 1–3*) or with GH for 5 (*lanes 4–6*), 30 (*lanes 7–9*), or 60 min (*lanes 10–12*) were analyzed by EMSA using a probe with the C/EBP site of the promoter of the *aP2* gene. The indicated antisera specific for phosphorylated C/EBPβ (Pβ) or C/EBPβ (β) were preincubated with nuclear extracts prior to EMSA. Similar results were obtained in three independent experiments.

may thus reflect hLAPT235A being present in forms with fewer phosphate groups compared with hLAP.

Phosphorylated C/EBPβ Is Transiently Bound to DNA—Because GH promotes a rapid MAPK-dependent phosphorylation of C/EBPβ, we examined whether complexes bound to DNA contain phosphorylated C/EBPβ. Nuclear extracts from 3T3-F442A cells treated with GH for different periods of time were analyzed by EMSA using a probe based on a well characterized C/EBP site from the *aP2* gene, which gave similar binding profiles to the c-*fos* C/EBP site in previous studies (34). In untreated cells, relatively low but detectable binding of LAP·LAP and LAP·LIP complexes is observed (Fig. 8, *lane 1*); these bands supershift in the presence of anti-C/EBPβ (*lane 3*). Only a slight supershift occurs with anti-P-C/EBPβ (*lane 2*), indicating that little if any of the LAP and LIP bound to DNA is phosphorylated at the MAPK site in untreated cells. In contrast, GH treatment of the cells for only 5 min leads to a substantial increase in the presence of phosphorylated LAP in supershifted complexes (*lane 5 versus 2, arrowhead*). In fact, the LAP·LAP and LAP·LIP complexes completely supershift in the presence of anti-P-C/EBPβ (*lane 5, arrowhead*), indicating that phosphorylated LAP and LIP are major components of the C/EBPβ complexes bound to DNA upon GH treatment of the cells. This is associated with a slight increase in binding of LAP·LAP and LAP·LIP dimers (*lane 4 versus 1*), as indicated by supershift in the presence of anti-C/EBPβ (*lane 6*). The presence of the phosphorylated C/EBPβ in complexes bound to DNA, then gradually decreases after 30–60 min (*lanes 8* and *11, arrowhead*). Similar results are obtained using the c-*fos* C/EBP site as probe (data not shown). The presence of phosphorylated C/EBPβ in complexes bound to DNA is consistent with the timing of GH-dependent phosphorylation of mLAP and mLIP at the MAPK site detected by SDS-PAGE and IEF (Fig. 2, *B* and *C*).

In addition, GH promotes a gradual increase in binding of LAP complexes by 30 and 60 min of GH treatment (Fig 8, *lanes 7* and *10*), and addition of anti-C/EBPβ reveals a corresponding increase in the supershift of LAP and LIP complexes (Fig. 8, *lanes 9* and *12*), as previously shown (34). This increase in binding of C/EBPβ complexes corresponds with the GH-promoted dephosphorylation of LAP (Fig. 1*B*), which increases binding as previously reported (5). Thus, endogenous C/EBPβ shows dynamic changes in DNA binding in response to GH that reflect both the transient phosphorylation at the MAPK site and subsequent dephosphorylation. These changes may determine the transient on-off nature of c-*fos* expression as influenced by GH and many other stimuli.

DISCUSSION

Dynamic Regulation of C/EBPβ Phosphorylation State Is Mediated by GH—These studies demonstrate for the first time that C/EBPβ is present in cells in multiple phosphorylated forms that are dynamically regulated. GH, as well as other growth factors, transiently increase phosphorylation of LAP, then promotes its dephosphorylation. IEF indicates that LAP is present in resting cells as multiple bands reflecting different degrees of phosphorylation. GH treatment rapidly (5–15 min) induces more acidic forms of LAP, consistent with increased phosphorylation. More basic bands appear within 30–60 min of GH treatment; these induced bands co-migrate with LAP dephosphorylated by alkaline phosphatase and represent GH-promoted dephosphorylation of LAP, as observed previously by conventional SDS-PAGE (5, 34). Both the phosphorylation and dephosphorylation of C/EBPβ occur in 3T3-F442A preadipocytes via endogenous GH receptors in response to physiological concentrations of GH. Similar changes in LAP and LIP phosphorylation also occur in response to other growth factors. For example, insulin-like growth factor-1 was found to promote both phosphorylation and dephosphorylation of mLAP and mLIP, and platelet-derived growth factor to stimulate their phosphorylation. The functional consequences of each of the regulated phosphorylation and dephosphorylation events are presumably integrated in the ultimate influence of C/EBPβ on gene transcription, in the present case, regulation of the c-*fos* promoter in response to GH. Dynamic regulation of transcription factor function by phosphorylation and dephosphorylation has similarly been reported for activation of AP-1 (4, 47).

MAPK Mediates Phosphorylation of C/EBPβ in Response to GH—The present studies demonstrate that C/EBPβ is a nuclear target for GH-stimulated MAPK activity. GH-stimulated ERK1/2 activity also mediates the phosphorylation of Elk-1, another transcription factor that is essential to trigger transcription via the c-*fos* serum response element (SRE) in response to GH (12). C/EBPβ contains phosphorylation sites for multiple kinases (5, 7, 8, 26–30). This is the first report showing that *in vivo* phosphorylation of Thr^{188} of mLAP and Thr^{37} of mLIP in the MAPK site are regulated by a physiological hormonal stimulus. These studies further show that C/EBPβ phosphorylation, even at this particular site, is complex. Immunoblotting with anti-P-C/EBPβ of proteins resolved by IEF shows that phosphorylated Thr^{188} is present in multiple forms of mLAP with different degree of phosphorylation (Fig. 2*B*). Changes in the level of the different phosphorylated forms of LAP may ultimately result in a dynamic regulation in the functions of this transcription factor, such as dimerization, DNA binding, interaction with other factor(s), and/or changes in transcriptional capacity. The presence of different phosphorylated forms of LAP may account for its versatility of function. For example, a recent report shows that phosphorylation of Thr^{217} of mLAP (Ser^{105} in rat LAP) by $p90^{rsk}$ creates a functional *XEXD* domain that enables LAP to function as a caspase substrate inhibitor (48).

The stimulation of LAP and LIP phosphorylation by GH and other growth factors parallels their ability to stimulate ERK1 and ERK2. Furthermore, the rapid phosphorylation of LAP at the MAPK site is necessary for GH-stimulated c-*fos* promoter activation. The same site (Thr^{235}) on hLAP, when phosphorylated *in vitro* by ERK-2, is reported to promote the interaction of hLAP with serum response factor (26). Serum response factor is critical for c-*fos* promoter activation in response to GH as well as other factors (11, 49). Thus, phosphorylation of C/EBPβ

at the conserved MAPK site, and/or at other site(s), may facilitate the interaction of C/EBPβ with serum response factor and/or other nuclear factors, such as TBP and TFIIB (50), NFκB family proteins (51, 52), the coactivator CBP/p300 (53), or SWI/SNF (54).

The GH-promoted phosphorylation of C/EBPβ at the MAPK site is rapid and transient (15–30 min), and GH-induced dephosphorylation, associated with inhibition of GSK-3 (5), appears to follow (30–60 min). The present studies indicate that the rapid phosphorylation of C/EBPβ at the MAPK site is necessary for GH-stimulated c-*fos* promoter activation. The subsequent dephosphorylation is better timed to contribute to the termination of c-*fos* promoter activation. Rapid and transient activation of c-*fos* expression is characteristic of this early-response gene in response to GH and many growth factors (35, 55, 56), and characteristically subsides within 60 min.

Changes in the Phosphorylation State of C/EBPβ Induced by GH Modulate Its DNA Binding and Transcriptional Activation—The present studies indicate that phosphorylation of one site (Thr^{188} in mLAP or Thr^{235} in hLAP) in conjunction with simultaneous phosphorylation at other sites may modulate C/EBPβ function. In fact, IEF shows a substantial decrease or even disappearance of certain phosphorylated forms of hLAPT235A in comparison to the bands of wild type hLAP (Fig. 7*D*). Mutation of Thr^{235} was reported to abrogate the constitutive phosphorylation of the nearby Ser^{231} in hLAP (7). It is possible that mutation of Thr^{235} would alter the phosphorylation of hLAP by other kinases or make hLAP more susceptible to dephosphorylation by endogenous phosphatases than wild type hLAP. Therefore, the presence of hLAPT235A in less phosphorylated forms might partially explain its higher DNA binding activity compared with wild type, possibly reflecting less charge repulsion between hLAP and DNA or differences in the conformation of LAP. The involvement of other sites of phosphorylation in addition to Thr^{235} in determining C/EBPβ DNA binding activity is further supported by the observation that alkaline phosphatase treatment of hLAPT235A increased its ability to bind to the c-*fos* or *aP2* C/EBP sites even more than the mutation alone. A restraining effect of phosphorylation on DNA binding activity has been described for other transcription factors of diverse nature, such as c-Jun (4), and members of nuclear receptor superfamily, such as the mineralocorticoid receptor (57, 58). With respect to C/EBPβ, several reports indicate that site selective DNA binding is attenuated by PKA and/or PKC mediated *in vitro* phosphorylation of C/EBPβ on Ser^{240}, located within the DNA-binding domain, as well as PKA dependent phosphorylation of other closely situated sites (between Ser^{173} and Ser^{223}) (28, 59). Phosphorylation of mLAP by GSK-3 (Ser^{184}) also reduces the DNA binding activity of LAP (5). GH treatment results in a partial inhibition of GSK-3, favoring the dephosphorylation of mLAP, and in consequence increasing binding of LAP complexes to DNA. Other mechanisms for generation of dephosphorylated LAP/LIP may occur (*e.g.* phosphatase activation, biosynthetic intermediate), but their contributions have not yet been identified. It is possible that complexes already bound to DNA become phosphorylated upon MAPK activation, because almost all bound C/EBPβ detected within 5 min of GH treatment is phosphorylated at the MAPK site; if so LAP might become transcriptionally active as a consequence. Maximum DNA binding of C/EBPβ complexes is detected by 60 min of treatment, coincidently with C/EBPβ dephosphorylation (Fig. 8). As phosphorylation subsides, the increasing binding of less phosphorylated, hence less transcriptionally active forms of C/EBPβ may then lead to termination of transcription. Clearly, the regulation and functional importance of phosphorylation and dephosphorylation at multiple sites on C/EBPβ is dynamic and complex, as shown here for GH. Future challenges are to understand more fully how multiple phosphorylation events modulate the function of individual transcription factors such as C/EBPβ, and additionally whether phosphorylation plays a role in coordinating the combined influences of multiple transcription factors on a single promoter such as c-*fos* to modulate gene expression.

Acknowledgments—We thank Dr. Quinguan Ge for providing anti-P-C/EBPβ, Dr. L. Argetsinger, J. Huo, and J. Kurzer for review of the manuscript, Dr. T. Blackwell for advice on statistics, and B. Hawkins for assistance with preparation of the manuscript.

REFERENCES

1. Hunter, T., and Karin, M. (1992) *Cell* **70,** 375–387
2. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) *Science* **264,** 1415–1421
3. Ihle, J. N. (1996) *Cell* **84,** 331–334
4. Boyle, W. J., Smeal, T., Delize, L. H., Angel, P., Woodgett, J. R., Korin, M., and Hunter, T. (1991) *Cell* **64,** 573–584
5. Piwien-Pilipuk, G., Van Mater, D., Ross, S. E., MacDougald, O. A., and Schwartz, J. (2001) *J. Biol. Chem.* **276,** 19664–19671
6. Kwok, R. P. S., Laurance, M. E., Lundblad, J. R., Goldman, P. S., Shih, H., O'Connor, L. M., Marriott, S. J., and Goodman, R. H. (1996) *Nature* **380,** 642–646
7. Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T., and Akira, S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2207–2211
8. Trautwein, C., Caelles, C., van der Geer, P., Hunter, T., Karin, M., and Chojkier, M. (1993) *Nature* **364,** 544–547
9. Gille, J., Kortenjann, M., Thomas, O., Moomaw, C., Slaughter, C., Cobb, M., and Shaw, P. E. (1995) *EMBO J.* **14,** 951–962
10. Price, M. A., Cruzalegui, F. H., and Treisman, R. (1996) *EMBO J.* **15,** 6552–6563
11. Liao, J., Hodge, C. L., Meyer, D. J., Ho, P. S., Rosenspire, K. C., and Schwartz, J. (1997) *J. Biol. Chem.* **272,** 25951–25958
12. Hodge, C., Liao, J., Stofega, M., Guan, K., Carter-Su, C., and Schwartz, J. (1998) *J. Biol. Chem.* **273,** 31327–31336
13. Descombes, P., and Schibler, U. (1991) *Cell* **67,** 569–579
14. Cao, Z., Umek, R. M., and McKnight, S. L. (1991) *Genes Dev.* **5,** 1538–1552
15. Lin, F.-T., and Lane, M. D. (1992) *Genes Dev.* **6,** 533–544
16. Darlington, G. J., Ross, S. E., and MacDougald, O. A. (1998) *J. Biol. Chem.* **273,** 30057–30060
17. Diehl, A. M. (1998) *J. Biol. Chem.* **273,** 30843–30846
18. Scott, L. M., Civin, C. I., Rorth, P., and Friedman, A. D. (1992) *Blood* **80,** 1725–1735
19. Tanaka, T., Akira, S., Yoshida, K., Umemoto, M., Yoneda, Y., Shirafuji, N., Fujiwara, H., Suematsu, S., Yoshida, N., and Kishimoto, T. (1995) *Cell* **80,** 353–361
20. Robinson, G. W., Johnson, P. F., Hennighausen, L., and Sterneck, E. (1998) *Genes Dev.* **12,** 1907–1916
21. Pall, M., Hellberg, P., Brannstrom, M., Mikuni, M., Peterson, C. M., Sundfeldt, K., Norden, B., Hedin, L., and Enerback, S. (1997) *EMBO J.* **16,** 5273–5279
22. Sterneck, E., Tessarollo, L., and Johnson, P. F. (1997) *Genes Dev.* **11,** 2153–2162
23. Croniger, C., Trus, M., Lysek-Stupp, K., Cohen, H., Liu, Y., Darlington, G. J., Poli, V., Hanson, R. W., and Reshef, L. (1997) *J. Biol. Chem.* **272,** 26306–26312
24. Screpanti, I., Romani, L., Musiani, P., Modesti, A., Fattori, E., Lazzaro, D., Sellitto, C., Scarpa, S., Bellavia, D., and Lattanzio, G. (1995) *EMBO J.* **14,** 1932–1941
25. Chen, X., Liu, W., Ambrosino, C., Ruocco, M. R., Poli, V., Romani, L., Quinto, I., Barbieri, S., Holmes, K. L., Venuta, S., and Scala, G. (1997) *Blood* **90,** 156–164
26. Hanlon, M., Sturgill, T. W., and Sealy, L. (2001) *J. Biol. Chem.* **276,** 38449–38456
27. Wegner, M., Cao, Z., and Rosenfeld, M. G. (1992) *Science* **256,** 370–373
28. Trautwein, C., van der Geer, P., Karin, M., Hunter, T., and Chojkier, M. (1994) *J. Clin. Invest.* **93,** 2554–2561
29. Chinery, R., Brockman, J. A., Dransfield, D. T., and Coffey, R. J. (1997) *J. Biol. Chem.* **272,** 30356–30361
30. Buck, M., Poli, V., van der Geer, P., Chojkier, M., and Hunter, T. (1999) *Mol. Cell* **4,** 1087–1092
31. Kowenz-Leutz, E., Twamley, G., Ansieau, S., and Leutz, A. (1994) *Genes Dev.* **8,** 2781–2791
32. Williams, S. C., Baer, M., Dillner, A. J., and Johnson, P. F. (1995) *EMBO J.* **14,** 3170–3183
33. Clarkson, R. W. E., Chen, C. M., Harrison, S., Wells, C., Muscat, G. E. O., and Waters, M. J. (1995) *Mol. Endocrinol.* **9,** 108–120
34. Liao, J., Piwien-Pilipuk, G., Ross, S. E., Hodge, C. L., Sealy, L., MacDougald, O. A., and Schwartz, J. (1999) *J. Biol. Chem.* **274,** 31597–31604
35. Muller, R. (1986) *Biochim. Biophys. Acta* **823,** 207–225
36. Schwartz, J., and Carter-Su, C. (1988) *Endocrinology* **122,** 2247–2256
37. Billestrup, N., Allevato, G., Norstedt, G., Moldrup, A., and Nielsen, J. H. (1994) *Proc. Soc. Exp. Biol. Med.* **206,** 205–209
38. Gong, T.-W. L., Meyer, D. J., Liao, J., Hodge, C. L., Campbell, G. S., Wang, X., Billestrup, N., Carter-Su, C., and Schwartz, J. (1998) *Endocrinology* **139,** 1863–1871
39. Harvat, B. L., and Wharton, W. (1995) *Cell Growth Differ.* **6,** 955–964
40. Christy, R. J., Yang, V. W., Ntambi, J. M., Geiman, D. E., Landschulz, W. H.,

Friedman, A. D., Nakabeppu, Y., Kelly, T. J., and Lane, M. D. (1989) *Genes Dev.* **3,** 1323–1335
41. Chen, D., and Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752
42. Campbell, G. S., Pang, L., Miyasaka, T., Saltiel, A. R., and Carter-Su, C. (1992) *J. Biol. Chem.* **267,** 6074–6080
43. Winston, L. A., and Bertics, P. J. (1992) *J. Biol. Chem.* **267,** 4747–4751
44. Moller, C., Hansson, A., Enberg, B., Lobie, P. E., and Norstedt, G. (1992) *J. Biol. Chem.* **267,** 23403–23408
45. VanderKuur, J. A., Butch, E. R., Waters, S. B., Pessin, J. E., Guan, K.-L., and Carter-Su, C. (1997) *Endocrinology* **138,** 4301–4307
46. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) *Biochem. J.* **351,** 95–105
47. Karin, M., Liu, Z., and Zandi, E. (1997) *Curr. Opin. Cell Biol.* **9,** 240–246
48. Buck, M., Poli, V., Hunter, T., and Chojkier, M. (2001) *Mol. Cell* **8,** 807–816
49. Treisman, R. (1992) *Trends Biochem. Sci.* **17,** 423–426
50. Nerlov, C., and Ziff, E. B. (1995) *EMBO J.* **14,** 4318–4328
51. Ruocco, M. R., Chen, X., Ambrosino, C., Dragonetti, E., Liu, W., Mallardo, M., De Falco, G., Palmieri, C., Franzoso, G., Quinto, I., Venuta, S., and Scala, G. (1996) *J. Biol. Chem.* **271,** 22479–22486
52. Stein, B., Cogswell, P. C., and Baldwin, A. S., Jr. (1993) *Mol. Cell. Biol.* **13,** 3964–3974
53. Mink, S., Haenig, B., and Klempnauer, K. H. (1997) *Mol. Cell. Biol.* **17,** 6609–6617
54. Kowenz-Leutz, E., and Leutz, A. (1999) *Mol. Cell* **4,** 735–743
55. Gurland, G., Ashcom, G., Cochran, B. H., and Schwartz, J. (1990) *Endocrinology* **127,** 3187–3195
56. Doglio, A., Dani, C., Grimaldi, P., and Ailhaud, G. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 1148–1152
57. Piwien-Pilipuk, G., and Galigniana, M. D. (1998) *Mol. Cell. Endocrinol.* **144,** 119–130
58. Galigniana, M. D. (1998) *Biochem. J.* **333,** 555–563
59. Mahoney, C. W., Shuman, J., McKnight, S. L., Chen, H. C., and Huang, K. P. (1992) *J. Biol. Chem.* **267,** 19396–19403