THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 41, Issue of October 10, pp. 25913–25919, 1997
Printed in U.S.A.

Signaling Pathways through Which Insulin Regulates CCAAT/Enhancer Binding Protein α (C/EBPα) Phosphorylation and Gene Expression in 3T3-L1 Adipocytes

CORRELATION WITH GLUT4 GENE EXPRESSION*

(Received for publication, July 11, 1997)

Nahid Hemati, Sarah E. Ross‡, Robin L. Erickson, Guy E. Groblewski, and Ormond A. MacDougald§

From the Department of Physiology, The University of Michigan Medical Center, Ann Arbor, Michigan 48109-0622

Treatment of 3T3-L1 adipocytes with insulin (IC_{50} ~200 pM insulin) or insulin-like growth factor-1 (IC_{50} ~200 pM IGF-1) stimulates dephosphorylation of CCAAT/enhancer binding protein α (C/EBPα), a transcription factor involved in preadipocyte differentiation. As assessed by immunoblot analysis of one- and two-dimensional PAGE, insulin appears to dephosphorylate one site within p30C/EBPα and an additional site within p42C/EBPα. Consistent with insulin causing dephosphorylation of C/EBPα through activation of phosphatidylinositol 3-kinase, addition of phosphatidylinositol 3-kinase inhibitors (*e.g.* wortmannin) blocks insulin-stimulated dephosphorylation of C/EBPα. In the absence of insulin, wortmannin or LY294002 enhance C/EBPα phosphorylation. Similarly, blocking the activity of FKBP-rapamycin-associated protein with rapamycin increases phosphorylation of C/EBPα in the absence of insulin. Dephosphorylation of C/EBPα by insulin is partially blocked by rapamycin, consistent with a model in which activation of FKBP-rapamycin-associated protein by phosphatidylinositol 3-kinase results in dephosphorylation of C/EBPα. The dephosphorylation of C/EBPα by insulin, in conjunction with the insulin-dependent decline in C/EBPα mRNA and protein, has been hypothesized to play a role in repression of GLUT4 transcription by insulin. Consistent with this hypothesis, the decline of GLUT4 mRNA following exposure of adipocytes to insulin correlates with dephosphorylation of C/EBPα. However, the repression of C/EBPα mRNA and protein levels by insulin is blocked with an inhibitor of the mitogen-activated protein kinase pathway without blocking the repression of GLUT4 mRNA, thus dissociating the regulation of C/EBPα and GLUT4 mRNAs by insulin. A decline in C/EBPα mRNA and protein may not be required to suppress GLUT4 transcription because insulin also induces expression of the dominant-negative form of C/EBPβ (liver inhibitory protein), which blocks transactivation by C/EBP transcription factors.

The 3T3-L1 cell line has been widely used to delineate the mechanisms through which insulin regulates gene expression and metabolism. (1–6). When exposed to appropriate differentiation stimuli, 3T3-L1 preadipocytes lose their fibroblastic features, undergo two cycles of mitosis, round-up, and acquire the morphological and biochemical phenotype of adipocytes. Although our understanding of the sequence of events involved in differentiation remains incomplete, compelling evidence indicates that differentiation occurs when forces that repress differentiation are overcome by forces that stimulate it (reviewed in Refs. 7–12). One of the transcription factors identified as playing a critical role in stimulating preadipocyte differentiation is CCAAT/enhancer binding protein α (C/EBPα).[1]

C/EBPα was purified and cloned as a hepatic nuclear factor that binds to the CCAAT motif and "core homology" sequences found in certain viral promoters/enhancers, as well as the albumin gene promoter (reviewed in Ref. 13). Following the cloning of the C/EBPα gene, other members of this gene family, including C/EBPβ and C/EBPδ, were identified and characterized. Members of the C/EBP family share considerable amino acid sequence identity within the C-terminal domain, which confers the ability to bind DNA (basic region) and to form dimers (leucine zipper) with themselves or with other family members. In contrast, C/EBP family members share little sequence identity in the N-terminal section, which contains the domains that activate or inhibit transcription.

Both C/EBPα and C/EBPβ are subject to translation from alternative start sites. For example, translation is initiated predominantly from the 1st and 3rd start codons in the C/EBPα mRNA, resulting in C/EBPα isoforms with molecular masses of 42 and 30 kDa, respectively (14, 15). Both p42C/EBPα and p30C/EBPα are capable of transactivating promoters that contain C/EBP-binding sites (14, 16). The C/EBPβ mRNA is also translated from the 1st and 3rd start codons giving rise to an active form, *l*iver *a*ctivator *p*rotein (LAP) (17), and a dominant-negative form, *l*iver *i*nhibitory *p*rotein (LIP) (18). Expression of LIP inhibits transactivation by other C/EBP transcription factors.

Although C/EBPα plays a critical role in preadipocyte differentiation, the importance of C/EBPα in mature adipocytes is unknown. Expression of C/EBPα remains high in adipocytes in culture and *in vivo*, suggesting that C/EBPα continues to regulate expression of adipocyte genes in these contexts. If so, the

* This work was supported in part by a research grant from the NIDDK, National Institutes of Health (RO1-DK51562) (to O. A. M.) 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.

‡ Recipient of a Predoctoral Fellowship from the National Science and Engineering Research Council of Canada.

§ Dept. of Physiology, University of Michigan Medical School 1301 E. Catherine Rd., Ann Arbor, MI 48109-0622. Tel.: 313-647-4880; Fax: 313-936-8813; E-mail: macdouga@umich.edu.

[1] The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; FRAP, FKBP-rapamycin-associated protein; GLUT4, insulin-responsive glucose transporter; IGF-1, insulin-like growth factor-1; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; MAPK, mitogen-activated protein kinase; PVDF, polyvinylidene difluoride.

regulation of C/EBPα by extracellular signals may coordinate transcription of a set of adipocyte genes, resulting in altered adipocyte gene expression and metabolism. A growing body of evidence indicates that C/EBPα may play a role in hormonal control of gene expression in adipocytes and other cell types (19–23). For example, the transcription of C/EBPα and C/EBPδ is rapidly and reciprocally regulated by glucocorticoids in 3T3-L1 adipocytes and white adipose tissue (24), consistent with other studies, which also implicate C/EBP transcription factors as playing a role in the effects of glucocorticoids on gene expression (19, 25, 26).

In addition to regulation by glucocorticoids, the expression of the C/EBP transcription factors is under rapid and dynamic regulation by insulin in fully differentated 3T3-L1 adipocytes. We have reported (21) that insulin regulates C/EBPα through at least three mechanisms: rapid dephosphorylation of C/EBPα, suppression of C/EBPα gene transcription, and inactivation of C/EBPα through induction of the dominant-negative LIP. C/EBPα, in turn, may then regulate the expression of adipocyte genes such as GLUT4, the insulin-responsive glucose transporter. Consistent with GLUT4 being regulated by C/EBPα in fully differentiated adipocytes, the promoter of GLUT4 contains a functional C/EBP-binding site (27) and induction of GLUT4 during preadipocyte differentiation is dependent upon expression of C/EBPα (28, 29). Moreover, the decline of GLUT4 following exposure of adipocytes to insulin (30) is temporally correlated with the suppression of C/EBPα mRNA and protein, the dephosphorylation of C/EBPα, and the induction of LIP (21). Taken together, these data are consistent with a model in which insulin-mediated regulation of C/EBP transcription factors results in repression of GLUT4 transcription.

The current study investigates the signaling pathways by which insulin regulates C/EBPα and other C/EBP family members and correlates these results with suppression of GLUT4. Addition of insulin to adipocytes stimulates dephosphorylation of C/EBPα through wortmannin- and rapamycin-sensitive signaling molecules, suggesting the involvement of phosphatidylinositol (PI) 3-kinase and FKBP-rapamycin-associated protein (FRAP) (31) in this process. In addition, the rapid decline in the amount of C/EBPα mRNA and protein is blocked by an inhibitor of the mitogen-activated protein kinase (MAPK) pathway. We demonstrate that suppression of GLUT4 mRNA by insulin does not require a decline in C/EBPα mRNA and protein, perhaps indicating that dephosphorylation of C/EBPα and induction of LIP play important roles in the regulation of GLUT4 by insulin.

EXPERIMENTAL PROCEDURES

Cell Culture—3T3-L1 preadipocytes were maintained and induced to differentiate into adipocytes as described previously (32), except that insulin was withdrawn from the medium on day 4 (24, 33). Fully differentiated 3T3-L1 adipocytes, 11–14 days after induction of differentiation, were switched to fresh media (either serum-free or containing 10% fetal bovine serum) 16–24 h before subjecting cells to treatment. Insulin (Sigma) was dissolved in 0.02 M HCl. Human recombinant insulin-like growth factor-1 (IGF-1) was obtained from Pharmacia Biotech Inc. in an aqueous solution. MEK inhibitor (PD98059; Parke-Davis), wortmannin (Sigma), LY294002 (BioMol Research Labs. Inc.), rapamycin (BioMol), and FK506 (BioMol) were dissolved in Me_2SO. The final concentration of vehicle was ≤0.1%.

Analysis of RNA—Cellular RNA was isolated from 3T3-L1 cells using RNA Stat60 (Tel-Test "B," Inc). The amount of C/EBPα, C/EBPβ, C/EBPδ, and GLUT4 mRNAs was assessed by Northern blot analysis (21, 24, 34). Total RNA (20 μg) was separated by electrophoresis in horizontal 1.2% agarose gels containing 6.5% formaldehyde. RNA was transferred to nylon membranes (Magna; Micron Separations Inc.) and covalently bonded to the membrane by exposure to ultraviolet light (1200 J/cm^2). Blots were prehybridized for 30 min (60 °C) in ExpressHyb (CLONTECH) and hybridized for 60 min at 60 °C in an identical solution containing 2×10^6 dpm of labeled probe per ml. Hybridized blots were washed three times in a solution containing 0.1 × SSC, 0.1% SDS at 65 °C. Autoradiography was at −80 °C with Kodak X-OMAT AR film (Eastman Kodak Co.) and an intensifying screen. Results were quantified using a PhosphorImager (Bio-Rad).

The DNA fragment used as a probe for C/EBPα mRNA was a ~900-base pair *Sac*I/*Hin*dIII fragment complementary to the 3′ end of the C/EBPα coding region, as well as part of the 3′-untranslated region (+1175 to +2078 nucleotides relative to transcriptional start site). The cDNA fragment for C/EBPβ is full length and was cloned from a 3T3-L1 adipocyte library as reported previously (35). The cDNA fragment used as a probe for C/EBPδ mRNA was as described (36). The cDNA fragment for GLUT4 was described previously (37). Isolated probes were labeled to high specific activity (~1×10^9 dpm per μg) by random hexamer priming (38).

Cell Lysates and Immunoblotting—3T3-L1 adipocyte monolayers (10-cm plates) were washed once with 10 ml of phosphate-buffered saline and scraped in 1 ml of a lysis buffer containing 1% SDS, 60 mM Tris-Cl, pH 6.8. Alternatively, monolayers from 6-cm Petri plates were lysed in 0.4 ml of lysis buffer. Lysates were boiled for 3 min, vortexed, and then boiled for an additional 7 min prior to storing at −21 °C. Western analysis was performed essentially as described previously (28). Briefly, proteins were separated by electrophoresis in 10.5% or 15% polyacrylamide gels (34). Protein was transferred to PVDF-Plus (Micron Separations, Inc.) at 150 mA overnight in a buffer containing 20% methanol, 25 mM Tris, and 0.2 M glycine. Filters were then blocked in 1% casein in 1 × TBS (25 mM Tris, 0.15 M NaCl, pH 7.6) (Pierce) for 1 h at room temperature. After a brief wash in 1 × TTBS (25 mM Tris, 150 mM NaCl, 0.05% Tween 20, 0.17% concentrated HCl, and 0.001% Thimerosol), filters were incubated with antisera or affinity purified antibody in a 1% bovine γ- globulin-1 × TTBS solution for 2 h at room temperature. After washing three times (10 min each) in 1 × TTBS, filters were incubated in 1 × TTBS containing 1% non-fat dried milk and a 1:4000 dilution of anti-rabbit IgG peroxidase conjugate (Sigma ImmunoChemicals) for 1 h at room temperature. Filters were washed as above prior to visualization of bound IgG-peroxidase using Super Signal or Super Signal Ultra Chemiluminescence Substrates (Pierce).

Antibodies—Immune serum against a synthetic peptide corresponding to an internal amino acid sequence of C/EBPα (present in both p42C/EBPα and p30C/EBPα) was prepared as described previously (14). Immune sera against synthethic peptides corresponding to internal amino acid sequences in C/EBPβ and C/EBPδ were described previously (21). Antisera used for Western blots are specific for each C/EBP transcription factor and interactions with other C/EBP isoforms are not observed.

Two-dimensional Gel Electrophoresis—Isoelectric focusing and SDS-PAGE were performed essentially as described previously (39) using the ISO-DALT system (Hoefer Scientific Instruments) and the method of O'Farrell (40). Forty-five min after the indicated hormonal treatment, 3T3-L1 adipocytes (6-cm plates) were lysed in 300 μl of a solution containing 9 M urea, 4% Nonidet P-40, and 1% β-mercaptoethanol and frozen at −21 °C. Isoelectric focusing was at room temperature for 16 h over a pH range of 3–10. Cellular proteins were separated by SDS-PAGE (12% acrylamide) at 10 °C for 16 h. Prestained molecular weight markers (Life Technologies, Inc.) and whole cell lysate samples were included in separate lanes as positive controls for M_r separation and immunoblot analysis, respectively. Transfer of protein to PVDF-Plus and immunoblot analysis were as described above.

RESULTS

Insulin Regulates C/EBPα Dephosphorylation through Activation of the Insulin Receptor—We have shown previously that high concentrations of insulin stimulate an increase in mobility of p30C/EBPα and p42C/EBPα on SDS-PAGE (21). To assess whether insulin regulates mobility of C/EBPα through activation of the insulin receptor, we examined the dependence of this effect on concentration of insulin. 3T3-L1 adipocytes were incubated with 0–300 nM insulin prior to cell lysis. Based on a previous time course (21), adipocytes were treated with insulin for 45 min. Upon immunoblot analysis (Fig. 1*A*), p42C/EBPα was observed as a broad diffuse band and mobility changes in p42C/EBPα were not observed. A minor band of slightly higher mobility than p42C/EBPα can be observed in this and other figures. This band corresponds to translation from the second in-frame methionine and gives rise to a protein of approxi-

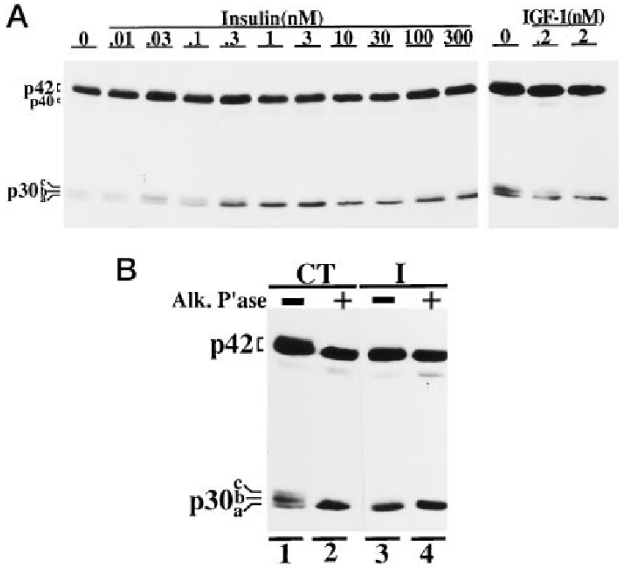

FIG. 1. *A*, effect of insulin or IGF-1 on mobility of C/EBPα during SDS-PAGE. The indicated concentrations of insulin or IGF-1 were added to 3T3-L1 adipocytes. After a 45-min incubation, whole cell lysates were prepared and subjected to immunoblot analysis for C/EBPα. The migration of p42, p40, and p30 forms of C/EBPα are indicated. p30C/EBPα migrates as three bands (*a-c*). Results from the insulin concentration dependence are representative of three independent experiments. The IGF-1 experiment was performed twice. *B*, mobility shift in C/EBPα is due to phosphorylation. Whole cell lysates were prepared from fully differentiated adipocytes (*CT*, *lanes 1* and *2*) or adipocytes treated with 167 nM insulin (*I*, *lanes 3* and *4*) for 45 min. Approximately 200 μg of protein from whole cell lysates was incubated (+, *lanes 2* and *4*) or not (−, *lanes 1* and *3*) with 100 units of alkaline phosphatase at 37 °C for 30 min followed by another 100 units for an additional 30 min. Proteins were then separated by SDS-PAGE, and the mobility of C/EBPα was observed by immunoblot analysis. Similar results were observed in four independent experiments.

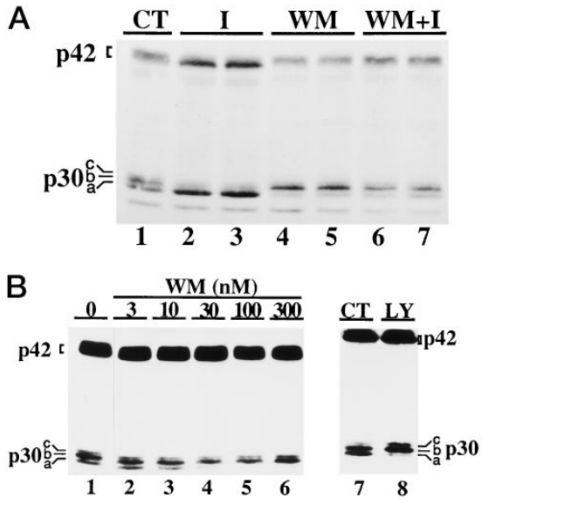

FIG. 2. **Induction of C/EBPα phosphorylation by wortmannin and LY294002.** *A*, 3T3-L1 adipocytes were incubated with vehicle (*CT*, *lane 1*), 167 nM insulin (*I*, *lanes 2* and *3*), 200 nM wortmannin (*WM*, *lanes 4* and *5*), or both 167 nM insulin and 200 nM wortmannin (*WM+I*, *lanes 6* and *7*) for 45 min. Whole cell lysates were immunoblotted with antibody against C/EBPα. *B*, concentration dependence of C/EBPα band pattern on wortmannin. 3T3-L1 adipocytes were incubated with 0–300 nM wortmannin for 45 min prior to immunoblot analysis for C/EBPα. Similar results were observed in eight independent experiments. In a separate experiment, 3T3-L1 adipocytes were not treated (*CT*, *lane 7*) or treated with 10 μM LY294002 (*LY*, *lane 8*) for 45 min before lysis and immunoblot analysis. Mobility of p42C/EBPα and the three bands of p30C/EBPα (*a-c*) are indicated. Similar results were observed in three independent experiments.

mately 40 kDa (Fig. 1*A*, *p40*). Translation from the third in-frame methionine gives rise to p30C/EBPα (14, 15). In lysates from nontreated adipocytes, p30C/EBPα migrates as three bands (Fig. 1*A*), with bands a and b expressed more highly than band c. Insulin causes p30C/EBPα to migrate almost exclusively as band a, the form that migrates most rapidly on SDS-PAGE. The concentration of insulin that elicited a half-maximal mobility shift in p30C/EBPα was between 0.1 and 0.3 nM. This concentration dependence correlates well with the K_D of 0.23 nM for the binding of insulin to the insulin receptor (41). Therefore, activation of the insulin receptor by insulin results in the loss of low mobility forms of p30C/EBPα with a concomitant increase in the high mobility form.

IGF-1 also stimulates a mobility shift in C/EBPα isolated from 3T3-L1 adipocytes. When adipocytes are treated with IGF-1 for 45 min, the generation of the high mobility form (Fig. 1*A*, *band a*) of p30C/EBPα is observed with 2 nM IGF-1. The concentration of IGF-1 required to give a half-maximal response was approximately 0.2 nM (Fig. 1*A*). This correlates with the binding constant for IGF-1 to the IGF-1 receptor (K_D = 0.16 nM (41). Thus, IGF-1 stimulates the loss of lower mobility forms of C/EBP through activation of the IGF-1 receptor.

Mobility Shift of C/EBPα upon SDS-PAGE Is Phosphorylation-dependent—A mobility shift in the migration of protein when subjected to SDS-PAGE is often indicative of a change in the phosphorylation of the protein. To determine if insulin induces dephosphorylation of C/EBPα, whole cell lysates from control adipocytes or adipocytes treated with 167 nM insulin were prepared. Approximately 200 μg of each lysate was incubated with or without alkaline phosphatase for 1 h prior to SDS-PAGE and immunoblot analysis for C/EBPα. The low mobility forms (Fig. 1*B*, *bands b* and *c*) of p30C/EBPα observed in immunoblots from control adipocytes were greatly reduced with a concomitant increase in the high-mobility form (*band a*) of p30C/EBPα. The mobility of p30C/EBPα from insulin-treated adipocytes (predominantly *band a*) was not influenced by alkaline phosphatase treatment, suggesting that the high mobility band a of p30C/EBPα represents the dephosphorylated form. (Fig. 1*B*, compare *lanes 3* and *4*). A similar regulation of p42C/EBPα can also be observed, although the changes in mobility are less dramatic. The effects of alkaline phosphatase were blocked by addition of phosphatase inhibitors (1 mM sodium orthovanadate, 60 μM β-glycerol phosphate, and 5 mg/ml *p*-nitrophenyl phosphate), and phosphatase inhibitors alone did not influence mobility of C/EBPα (data not shown). These data indicate that the retarded mobility of C/EBPα upon SDS-PAGE is due to phosphorylation of C/EBPα. Therefore, the loss of lower mobility forms of C/EBPα after insulin or IGF-1 treatment is due to dephosphorylation of C/EBPα.

Insulin Regulates C/EBPα Dephosphorylation through Activation of PI 3-kinase—To ascertain whether the dephosphorylation of C/EBPα by insulin is mediated through PI 3-kinase, 3T3-L1 adipocytes were incubated with insulin (167 nM) and/or the PI 3-kinase inhibitor, wortmannin (200 nM), prior to cell lysis and immunoblot analysis. While insulin results in a predominantly high-mobility dephosphorylated form of p30C/EBPα (Fig. 2*A*, *band a*), wortmannin results in a lower mobility phosphorylated form (Fig. 2*A*, *band b*). Although less dramatic than with p30C/EBPα, this regulation is also observed with p42C/EBPα. As expected for an inhibitor of a downstream signaling pathway, wortmannin blocks the ability of insulin to stimulate dephosphorylation of C/EBPα. Indeed, wortmannin stimulates the phosphorylation of p30C/EBPα (*band b*) even when insulin is added simultaneously. An even lower mobility form of p30C/EBPα (*band c*) can also be observed, suggesting the presence of additional phosphorylated residues not regulated by wortmannin.

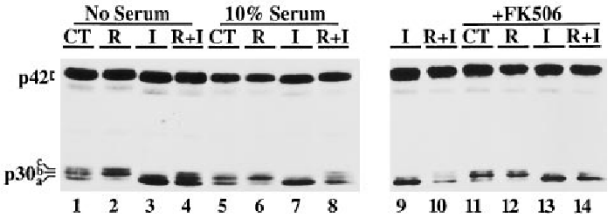

FIG. 3. **Induction of C/EBPα phosphorylation by rapamycin.** Sixteen h before treatments were applied, adipocyte medium was changed to DMEM (*No serum*, *lanes 1–4*) or DMEM with 10% fetal calf serum (*10% Serum, lanes 5–8*). 3T3-L1 adipocytes were incubated for 45 min with vehicle (*CT*, *lanes 1* and *5*), 40 nM rapamycin (*R*, *lanes 2* and *6*), 167 nM insulin (*I*, *lanes 3* and *7*) or with insulin after a 20-min pretreatment with rapamycin (*R+I*, *lanes 4* and *8*). Whole cell lysates were subjected to immunoblot analysis for C/EBPα. Mobility of p42C/EBPα and the three bands of p30C/EBPα (*a-c*) are indicated. In a separate experiment, adipocytes were incubated for 16 h in serum-free medium. Some adipocytes were not pretreated (*lanes 9* and *10*) or pretreated for 10 min with 5 μM FK506 (*lanes 11–14*). Adipocytes were then treated with vehicle (*CT*, *lane 11*), 5 nM rapamycin (*R*, *lane 12*), 40 nM insulin (*I*, *lanes 9* and *13*), or 5 nM rapamycin and 40 nM insulin (*R+I*, *lanes 10* and *14*). Similar results were observed in six independent experiments.

Since the concentration of wortmannin needed to block PI 3-kinase is much less than the concentrations needed to block other enzymes, the dependence of C/EBPα phosphorylation on concentration of wortmannin was examined (Fig. 2*B*, *lanes 1-6*). Wortmannin caused the phosphorylation of p30C/EBPα, with a concentration-dependent loss of the dephosphorylated form of p30C/EBPα (Fig. 2*B*, *band a*). The half-maximal effects of wortmannin on p30C/EBPα were observed between 10 and 30 nM, consistent with the concentration needed to block other effects of insulin mediated through PI 3-kinase in adipocytes, such as insulin-induced glucose uptake. To provide additional evidence that PI 3-kinase is involved in regulating C/EBPα phosphorylation, a PI 3-kinase inhibitor (LY294002) structurally unrelated to wortmannin was incubated with 3T3-L1 adipocytes for 45 min prior to preparation of whole cell lysates, SDS-PAGE, and immunoblot analysis for C/EBPα. LY294002, like wortmannin, results in the phosphorylation of C/EBPα, as assessed by the formation of band b (and to a lesser extent, band c) of p30C/EBPα (Fig. 2*B*, *lanes 7* and *8*). Taken together, these data indicate that PI 3-kinase activity is necessary for insulin to stimulate dephosphorylation of C/EBPα.

Insulin Regulates C/EBPα Dephosphorylation through a Rapamycin-sensitive Pathway—Part of the insulin-signaling pathway beyond PI 3-kinase is mediated by FRAP (also known as rapamycin and FKBP12 target 1, mammalian targets of rapamycin, and RAPT1 (31, 42–44)), activation of which is sufficient to induce $p70^{S6kinase}$ and $p85^{S6kinase}$ activities. The FRAP pathway is inhibited by rapamycin, which binds specifically to FK506-binding protein. The rapamycin·FK506 binding protein complex then binds and inhibits FRAP activity. To determine whether insulin-induced dephosphorylation of C/EBPα occurs through a rapamycin-sensitive pathway, fully differentiated adipocytes were incubated with vehicle (Fig. 3, *CT*), rapamycin (*R*), insulin (*I*), or rapamycin and insulin (*R+I*) and subjected to immunoblot analysis. We observed that the presence of serum correlated with a higher proportion of the dephosphorylated form of p30C/EBPα, and that incubation under serum-free conditions led to an increase in the phosphorylated forms. Thus, this experiment was performed after 16 h in serum-free medium (Fig. 3, *lanes 1–4*) or in the presence of 10% fetal calf serum (Fig. 3, *lanes 5–8*). Incubation of 3T3-L1 adipocytes in serum-containing medium increases the proportion of the highest mobility form of p30C/EBPα (*band a*) but does not substantially influence regulation of C/EBPα phosphorylation by insulin or rapamycin. Like wortmannin, rapamycin stimulates the formation of the phosphorylated forms of p30C/EBPα (Fig. 3, *band b* and, to a lesser extent, *band c*). Compared with wortmannin, rapamycin only partially blocks the ability of insulin to dephosphorylate C/EBPα (Fig. 3, *lanes 4* and *8*). Addition of 5 μM FK506 blocks the ability of rapamycin to inhibit insulin-induced dephosphorylation of C/EBPα (Fig. 3, *compare lanes 9* and *10* with *lanes 13* and *14*), indicating that rapamycin is acting through a drug-receptor complex involving a FK506-binding protein. The partial block of insulin-stimulated dephosphorylation of C/EBPα by rapamycin may indicate that activation of parallel rapamycin-dependent and -independent pathways is necessary for the complete dephosphorylation of C/EBPα.

Retarded Mobility of C/EBPα upon SDS-PAGE after Wortmannin or Rapamycin Treatment Is Due to Phosphorylation—To ascertain whether the formation of the lower mobility forms of p30C/EBPα by wortmannin or rapamycin is due to phosphorylation, adipocytes were treated with wortmannin or rapamycin, and cell lysates were prepared. Samples were incubated with or without alkaline phosphatase as described in Fig. 1*B*. Upon immunoblot analysis, the predominant form of p30C/EBPα upon SDS-PAGE of lysates from wortmannin- and rapamycin-treated adipocytes was band b (Fig. 4*A*, *lanes 1* and *3*). Phosphatase treatment caused the loss of bands b and c with an increase in the high mobility band a of p30C/EBPα. Therefore, the gain of low-mobility forms of p30C/EBPα after wortmannin- or rapamycin-treatment is due to increased phosphorylation of C/EBPα.

Regulation of C/EBPα Phosphorylation Alters Mobility of p42C/EBPα upon Two-dimensional PAGE—These results demonstrate that insulin induces dephosphorylation of p30C/EBPα, and inhibition of PI 3-kinase or RAFT/FRAP results in the accumulation of hyperphosphorylated forms of p30C/EBPα (Figs. 1–4*A*). To better resolve the comparable changes in p42C/EBPα, adipocytes treated with vehicle, insulin, wortmannin, or rapamycin were lysed and subjected to isoelectric focusing prior to SDS-PAGE and immunoblot analysis. Phosphorylation of proteins decreases their pI, and the mobility of the resulting phosphoprotein is shifted toward the acidic end of gels upon isoelectric focusing (45). Two-dimensional gel analysis of p42C/EBPα reveals at least seven complexes of different pI and slightly different M_r (Fig. 4*B*). This heterogeneous migration is consistent with that of other phosphoproteins (*e.g.* PHAS-1) (46). Such heterogeneity is also consistent with p42C/EBPα migrating as a broad, poorly resolved band upon SDS-PAGE (Figs. 1–4*A*). When compared with the pattern of p42C/EBPα in control lysates, insulin causes a basic shift in mobility of p42C/EBPα with complete loss of G and a decline in F with concomitant increases in forms within complex C and B (Fig. 4*B*). In contrast, when the pattern of p42C/EBPα in lysates from wortmannin- and rapamycin-treated adipocytes are compared with control, an acidic shift is observed in the mobility of p42C/EBPα. A decline is observed in the most basic complexes (*A* and *B*), with a slight increase observed in the most acidic complex (Fig. 4*B*, *G*). Thus, these observations are consistent with insulin stimulating dephosphorylation of p42C/EBPα and wortmannin or rapamycin stimulating phosphorylation of p42C/EBPα.

Insulin-dependent Dephosphorylation of C/EBPα Is Independent of MAPK—In addition to activating the PI 3-kinase pathway, insulin also activates the MAPK (ERK1 and 2) pathway. To determine whether MAPK activity is necessary for insulin to stimulate C/EBPα dephosphorylation, MAPK activity was inhibited by inhibiting MEK1 and MEK2, the enzymes immediately upstream of ERK1 and ERK2, with the MEK

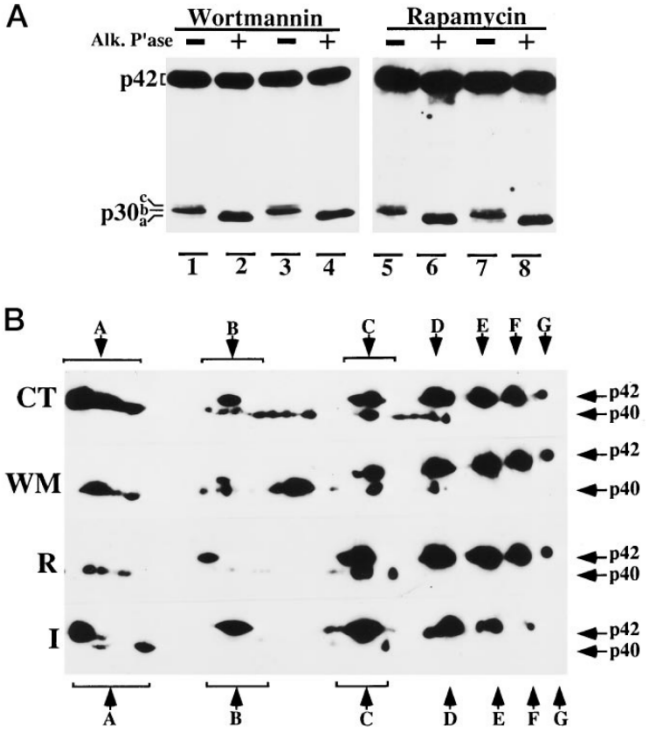

FIG. 4. *A*, mobility shift of C/EBPα after wortmannin or rapamycin treatment is due to phosphorylation. Whole cell lysates were prepared from adipocytes treated with 200 nM wortmannin (*lanes 1–4*) or 45 nM rapamycin (*lanes 5–8*) for 45 min. Approximately 200 μg of whole cell protein was incubated (*lanes 2, 4, 6,* and *8*) or not (*lanes 1, 3, 5,* and *7*) with 100 units of alkaline phosphatase at 37 °C for 30 min followed by another 100 units for an additional 30 min. Proteins were then separated by SDS-PAGE, and the mobility of C/EBPα was observed by immunoblot analysis. The migration of p42C/EBPα, and the three bands of p30C/EBPα (*a-c*) are indicated. *B*, immunoblot analysis of C/EBPα after two-dimensional separation of adipocyte lysate. Fully differentiated 3T3-L1 adipocytes were incubated with vehicle (*CT*), 100 nM wortmannin (*W*), 50 nM rapamycin (*R*), or 167 nM insulin (*I*) for 45 min prior to lysis and separation by isoelectric focusing and SDS-PAGE. After transfer to PVDF overnight, immunoblot analysis was performed for C/EBPα. The migrations of p42C/EBPα and p40C/EBPα are indicated. The basic forms of C/EBPα were designated *A* to *C* because of multiple forms within these complexes. The more acidic forms of C/EBPα appeared more homogeneous and are designated *D-G*. These results are representative of four samples analyzed during three independent experiments. Following wortmannin treatment, the *dark band* on the acidic side of *complex B* was not consistently observed.

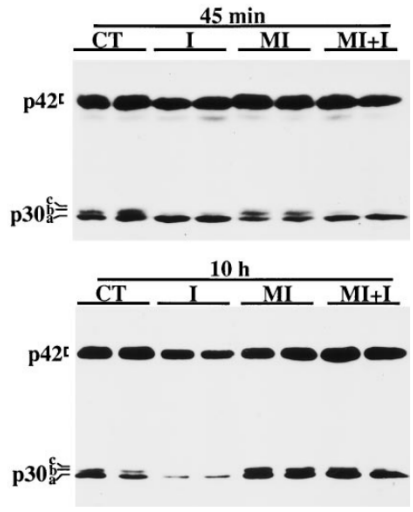

FIG. 5. **MEK inhibitor does not affect insulin-dependent dephosphorylation of C/EBPα but does block the insulin-induced suppression of C/EBPα protein.** 3T3-L1 adipocytes were incubated for 45 min or 10 h with vehicle (*CT*), 167 nM insulin (*I*), 40 μM PD98059 (MEK inhibitor, *MI*), or 167 nM insulin after a 20-min pretreatment with MEK inhibitor (*MI+I*). Whole cell lysates were prepared and subjected to immunoblot analysis for C/EBPα. Similar results were observed in three independent experiments.

inhibitor PD098059 (47). Immunoblot analysis with antiserum specific to activated MAPK (Promega) indicated that insulin activated MAPK within 10 min, and MAPK activation was undetectable in lysates from control adipocytes or in adipocytes in which insulin was added in the presence of 40 μM MEK inhibitor (data not shown). To investigate the effect on C/EBPα phosphorylation, fully differentiated 3T3-L1 adipocytes were incubated for 45 min with vehicle (Fig. 5, *CT*) insulin (*I*), MEK inhibitor (*MI*), or MEK inhibitor and insulin (*MI+I*). Adipocytes were then lysed and subjected to immunoblot analysis with antiC/EBPα antibody (Fig. 5). MEK inhibitor did not influence the ability of insulin to stimulate C/EBPα dephosphorylation. Moreover, the addition of MEK inhibitor alone had no effect on phosphorylation of C/EBPα. Thus, insulin does not appear to induce dephosphorylation of C/EBPα through activation of the MAPK pathway.

Insulin Suppresses C/EBPα Protein and mRNA Levels through Activation of MAPK—In addition to stimulating dephosphorylation of C/EBPα, insulin also inhibits transcription of the C/EBPα gene, which is followed by a decline in the amount of C/EBPα mRNA and protein (21). To ascertain whether MAPK activity is required for the suppression of the quantity of C/EBPα protein by insulin, fully differentiated 3T3-L1 adipocytes were incubated for 10 h with vehicle (Fig. 5, *CT*) insulin (*I*), MEK inhibitor (*MI*), or MEK inhibitor and insulin (*MI+I*). Adipocytes were then lysed and subjected to immunoblot analysis (Fig. 5). MEK inhibitor blocked the insulin-dependent decline in amount of C/EBPα protein, while addition of MEK inhibitor alone had no effect on expression of C/EBPα protein. These data indicate that activation of the MAPK (ERK1 and 2) pathway is required for suppression of the level of C/EBPα protein by insulin.

To further test the hypothesis that insulin regulates C/EBPα gene expression through activation of the MAPK pathway, experiments were performed to investigate whether MEK inhibitor blocks the suppression of C/EBPα mRNA levels by insulin. Total RNA was isolated from adipocytes treated with insulin (Fig. 5, *I*), MEK inhibitor (*MI*), or MEK inhibitor and insulin (*MI+I*) for 0, 2, or 6 h and subjected to Northern blot analysis. As observed previously (21), treatment of adipocytes with insulin represses the expression of C/EBPα mRNA at both 2 and 6 h. However, this inhibition of C/EBPα mRNA by insulin was completely blocked by MEK inhibitor (Fig. 6*A*), indicating that activation of MAPK activity is required for suppression of C/EBPα mRNA by insulin. Addition of MEK inhibitor alone consistently induced C/EBPα mRNA at 2 h by 10–20%, suggesting that basal activation of the MAPK pathway may be involved in regulating the base-line level of C/EBPα expression in 3T3-L1 adipocytes.

MEK Inhibitor Dissociates Coregulation of C/EBPα and GLUT4 mRNAs by Insulin—Because of the considerable evidence indicating that C/EBPα regulates the insulin-sensitive glucose transporter, GLUT4, we have hypothesized that insulin represses GLUT4 gene expression in part through causing a decline in C/EBPα mRNA and protein. If suppression of C/EBPα mRNA is necessary for suppression of GLUT4 by insulin, we would predict that, since inhibition of MEK maintains the expression of C/EBPα mRNA in the presence of insulin (Fig. 6*A*), inhibition of MEK would also maintain the expression of GLUT4. In sharp contrast to this hypothesis, inhibition of MEK had no effect on the insulin-induced decline in GLUT4 mRNA (Fig. 6*B*). These findings indicate that GLUT4 mRNA levels are

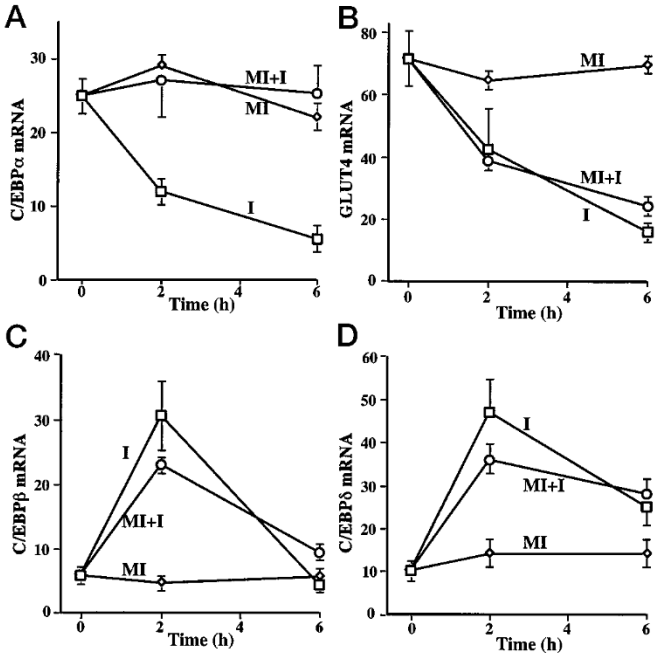

FIG. 6. **Effect of the MEK Inhibitor, PD98059, on the regulation of C/EBPα, GLUT4, C/EBPβ, and C/EBPδ mRNAs by insulin.** Fully differentiated 3T3-L1 adipocytes (14 days after induction of differentiation) were incubated for 2 or 6 h with 167 nM insulin (*I, square*), 40 μM PD98059 (MEK inhibitor, *MI*, *diamond*), or 167 nM insulin after a 20-min pretreatment with MEK inhibitor (*MI+I*, *circle*). Total RNA was prepared from nontreated adipocytes as well as from cells incubated for the indicated times. Northern blot analysis was performed with probes specific for C/EBPα (*A*), GLUT4 (*B*), C/EBPβ (*C*), and C/EBPδ (*D*). Results were quantified using a Bio-Rad PhosphorImager and are expressed as arbitrary absorbance units (mean ± range). The results from one experiment are represented graphically. Similar results were observed in five independent experiments.

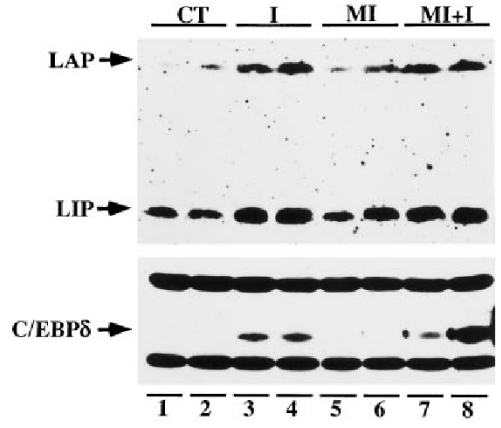

FIG. 7. **MEK inhibitor fails to block induction of C/EBPβ and C/EBPδ proteins by insulin.** 3T3-L1 adipocytes were incubated for four h with vehicle (*CT*), 167 nM insulin (*I*), 40 μM PD98059 (MEK inhibitor, *MI*), or 167 nM insulin after a 20-min pretreatment with MEK inhibitor (*MI+I*). Whole cell lysates were prepared and subjected to immunoblot analysis for C/EBPβ and C/EBPδ. The migration of C/EBPβ (LAP and LIP) and C/EBPδ proteins are indicated. Similar results were observed in two independent experiments.

not correlated with C/EBPα mRNA levels following insulin treatment, suggesting that it is the posttranslational modifications of C/EBPα by insulin that are critical for regulation of GLUT4. Alternatively, the induction by insulin of a dominant-negative form of C/EBP (*e.g.* LIP) could be involved in suppressing GLUT4 expression.

Induction of LIP Correlates with the Suppression of GLUT4 mRNA by Insulin—We demonstrated previously that insulin rapidly and transiently induces the expression of both C/EBPβ and C/EBPδ in 3T3-L1 adipocytes (21). To ascertain whether the regulation of these genes by insulin was influenced by inhibition of MEK, 3T3-L1 adipocytes were incubated for various times with 167 nM insulin, 40 μM MEK inhibitor, or a combination of MEK inhibitor and insulin prior to preparation of total RNA for Northern blot analysis and whole cell lysates for immunoblot analysis. As expected, insulin rapidly and transiently induces the expression of both C/EBPβ (Fig. 6*C*) and C/EBPδ (Fig. 6*D*). Furthermore, inhibition of MEK did not block the transient induction of either gene by insulin. Addition of MEK inhibitor alone had no effect on expression of C/EBPβ or C/EBPδ. Thus, activation of MAPK does not appear to be required for the transient induction of C/EBPβ and C/EBPδ mRNAs by insulin.

We demonstrated previously that the primary result of the insulin-dependent increase in C/EBPβ and C/EBPδ mRNAs is an increase in the dominant-negative form of C/EBPβ, LIP (21). Since induction of LIP by insulin is hypothesized to result in repression of C/EBP-responsive genes such as GLUT4, we determined whether the expression of LAP, LIP, and C/EBPδ was influenced by MEK inhibitor. 3T3-L1 adipocytes were incubated with insulin (Fig. 7, *I*), MEK inhibitor (*MI*), or MEK inhibitor and insulin (*MI+I*) for 4 h, and whole cell lysates were used for immunoblot analysis (Fig. 7). Consistent with the induction of C/EBPβ and C/EBPδ mRNA levels at 2 h, expression of LAP, LIP, and C/EBPδ proteins were induced at 4 h in adipocytes treated with insulin or MEK inhibitor and insulin. No regulation of C/EBPβ or C/EBPδ proteins was observed with MEK inhibitor alone. Consistent with our previous observation (21), LIP is more highly expressed than LAP in fully differentiated adipocytes. While insulin induces a proportionate increase in both forms of C/EBPβ, as well as C/EBPδ, the major result is an increase in quantity and binding activity of LIP (Fig. 7) (21). Since MAPK activity is not required for the induction of LIP by insulin, the increase in the expression of this dominant negative form of C/EBPβ is correlated with the decline in GLUT4 mRNA.

DISCUSSION

Insulin Induces Dephosphorylation of C/EBPα—The current study demonstrates that C/EBPα is a phosphoprotein in 3T3-L1 adipocytes and that sites within p30C/EBPα and p42C/EBPα are rapidly dephosphorylated following exposure of adipocytes to insulin or IGF-1 (Fig. 1). Separation of p42C/EBPα by two-dimensional PAGE indicates that there may be as many as seven sites of phosphorylation, of which two are dephosphorylated in response to insulin (Fig. 4*B*). One of these sites is located within p30C/EBPα, and an additional site is found within the N-terminal 12 kDa of p42C/EBPα. The role of phosphorylation in regulating the activities of C/EBPα is unknown; however, it likely plays an important role in adipocyte gene expression since phosphorylation plays an important regulatory role for other transcription factors (48).

We speculate that dephosphorylation of C/EBPα by insulin reduces the transactivational activity of C/EBPα and thereby results in decreased GLUT4 transcription. Thus, when insulin-induced dephosphorylation of C/EBPα is blocked with wortmannin (Fig. 2), the insulin-induced decline in GLUT4 mRNA observed at 2 h is greatly reduced (data not shown). Since C/EBPα autoactivates transcription from the C/EBPα promoter (14, 49), it is not surprising that we also observe a block of the insulin-dependent decline in C/EBPα mRNA (data not shown). Further investigation will be required to fully determine the role of insulin-dependent dephosphorylation of C/EBPα in the suppression of GLUT4 gene expression.

Stimulation of the insulin or IGF-1 receptor is sufficient to

cause C/EBPα dephosphorylation (Fig. 1). Because the abundance of IGF-1 receptors is small in adipocytes relative to the number of insulin receptors, the IGF-1 receptors likely exist as hybrids with the insulin receptor (50). If so, IGF-1 may stimulate C/EBPα dephosphorylation by activation of hybrid insulin-IGF-1 receptors.

Signaling Pathways by Which Insulin Regulates C/EBPα—Insulin regulates the phosphorylation and activity of many cytosolic proteins (*e.g.* enzymes involved in intermediary metabolism); however, relatively few nuclear targets have been identified. The sensitivity of insulin-induced dephosphorylation of C/EBPα to wortmannin (Fig. 2), rapamycin (Fig. 3), and okadaic acid (21) suggests that PI 3-kinase, FRAP, and protein phosphatase 1 or 2A, respectively, may play direct or indirect roles in the signaling pathway used by insulin to regulate C/EBPα kinase and/or phosphatase. The complex of rapamycin and FK506-binding protein activates FRAP, which then activates $p70^{S6kinase}$ and its nuclear-localized form, $p85^{S6kinase}$ (51). While the role of $p70^{S6kinase}$ in regulating the rate of translation is well known, the importance of $p85^{S6kinase}$ is less clear. One possibility is that $p85^{S6kinase}$ is involved in transducing the insulin signal to the nucleus, resulting in dephosphorylation of C/EBPα. With these signaling molecules identified as candidates, dominant-negative or constitutively-active constructs can now be used to test the role of these proteins as signaling intermediates more directly.

In addition to causing dephosphorylation of C/EBPα, insulin also stimulates a decline in C/EBPα mRNA and protein, a process dependent upon MAPK activity (Figs. 5 and 6). Because the decline in GLUT4 following insulin treatment occurs in the absence of MAPK activity (Fig. 6), a fall in C/EBPα mRNA and protein levels does not appear to be necessary for the regulation of GLUT4 by insulin. In contrast, the insulin-dependent dephosphorylation of C/EBPα correlates well with the suppression of GLUT4 mRNA levels since both occur by MAPK-independent mechanisms (Figs. 5 and 6). Similarly, the transient induction of C/EBPβ and C/EBPδ by insulin, which results in a large increase in LIP, also correlates with suppression of GLUT4 mRNA since both of these events are independent of MAPK activity (Fig. 6 and data not shown).

In summary, insulin represses expression of C/EBPα mRNA and protein through activation of the MAPK signaling pathway. In addition, insulin stimulates the dephosphorylation of C/EBPα through activation of the insulin receptor PI 3-kinase and FRAP. Finally, insulin greatly increases the expression of the dominant-negative form of C/EBPβ, LIP, with a mechanism independent of MAPK or PI 3-kinase. Insulin-stimulated dephosphorylation of C/EBPα, in conjunction with inactivation of C/EBPα by insulin-dependent induction of LIP may play a role in the repression of insulin-responsive adipocyte genes such as GLUT4.

Acknowledgments—The authors thank Lawrence Argetsinger, Christin Carter-Su, and Jessica Schwartz for critical review of this manuscript.

REFERENCES

1. Green, H., and Kehinde, O. (1974) *Cell* **1,** 113–116
2. Green, H., and Meuth, M. (1974) *Cell* **3,** 127–133
3. Green, H., and Kehinde, O. (1975) *Cell* **5,** 19–27
4. Coleman, R. A., Reed, B. C., Mackall, J. C., Student, A. K., Lane, M. D., and Bell, R. M. (1978) *J. Biol. Chem.* **253,** 7256–7261
5. Mackall, J. C., Student, A. K., Polakis, S. E., and Lane, M. D. (1976) *J. Biol. Chem.* **251,** 6462–6464
6. Mackall, J. C., and Lane, M. D. (1977) *Biochem. Biophys. Res. Commun.* **79,** 720–725
7. Cornelius, P., MacDougald, O. A., and Lane, M. D. (1994) *Annu. Rev. Nutr.* **14,** 99–129
8. Smas, C. M., and Sul, H. S. (1995) *Biochem. J.* **309,** 697–710
9. Spiegelman, B. M., and Flier, J. S. (1996) *Cell* **87,** 377–389
10. MacDougald, O. A., and Lane, M. D. (1995) *Annu. Rev. Biochem.* **64,** 345–373
11. MacDougald, O. A., and Lane, M. D. (1995) *Curr. Biol.* **5,** 618–621
12. Mandrup, S., and Lane, M. D. (1997) *J. Biol. Chem.* **272,** 5367–5370
13. McKnight, S. L. (1992) *Transcriptional Regulation*, pp. 771–795, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
14. Lin, F.-T., MacDougald, O. A., Diehl, A. M., and Lane, M. D. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9606–9610
15. Ossipow, V., Descombes, P., and Schibler, U. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8219–8223
16. Hwang, C.-S., Mandrup, S., MacDougald, O. A., Geiman, D. E., and Lane, M. D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 873–877
17. Descombes, P., Chojkier, M., Lichtsteiner, S., Falvey, E., and Schibler, U. (1990) *Genes Dev.* **4,** 1541–1551
18. Descombes, P., and Schibler, U. (1991) *Cell* **67,** 569–579
19. Gotoh, T., Chowdhury, S., Takiguchi, M., and Mori, M. (1997) *J. Biol. Chem.* **272,** 3694–3698
20. Roesler, W. J., Crosson, S. M., Vinson, C., and McFie, P. J. (1996) *J. Biol. Chem.* **271,** 8068–8074
21. MacDougald, O. A., Cornelius, P., Liu, R., and Lane, M. D. (1995) *J. Biol. Chem.* **270,** 647–654
22. O'Brien, R. M., Lucas, P. C., Yamasaki, T., Noisin, E., and Granner, D. K. (1994) *J. Biol. Chem.* **269,** 30419–30428
23. Park, E. A., Song, S., Olive, M., and Roesler, W. J. (1997) *Biochem. J.* **322,** 343–349
24. MacDougald, O. A., Cornelius, P., Lin, F.-T., Chen, S. S., and Lane, M. D. (1994) *J. Biol. Chem.* **269,** 19041–19047
25. Ratajczak, T., Williams, P. M., DiLorenzo, D., and Ringold, G. M. (1992) *J. Biol. Chem.* **267,** 11111–11119
26. Ramos, R. A., Nishio, Y., Maiyar, A. C., Simon, K. E., Ridder, C. C., Ge, Y., and Firestone, G. L. (1996) *Mol. Cell. Biol.* **16,** 5288–5301
27. Kaestner, K. H., Christy, R. J., and Lane, M. D. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 251–255
28. Lin, F.-T., and Lane, M. D. (1992) *Genes Dev.* **6,** 533–544
29. Lin, F.-T., and Lane, M. D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8757–8761
30. Flores-Riveros, J. R., McLenithan, J. C., Ezaki, O., and Lane, M. D. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 512–516
31. Brown, E. J., Albers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S., and Schreiber, S. L. (1994) *Nature* **369,** 756–758
32. Student, A. K., Hsu, R. Y., and Lane, M. D. (1980) *J. Biol. Chem.* **255,** 4745–4750
33. MacDougald, O. A., Hwang, C.-S., Fan, H., and Lane, M. D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 9034–9037
34. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
35. Potter, J. J., Mezey, E., Cornelius, P., Crabb, D. W., and Yang, V. W. (1992) *Arch. Biochem. Biophys.* **295,** 360–368
36. Cao, Z., Umek, R. M., and McKnight, S. L. (1991) *Genes Dev.* **5,** 1538–1552
37. Kaestner, K. H., Christy, R. J., McLenithan, J. C., Braiterman, L. T., Cornelius, P., Pekala, P. H., and Lane, M. D. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 3150–3154
38. Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13
39. Wishart, M. J., Groblewski, G., Goke, B. J., Wagner, A. C. C., and Williams, J. A. (1994) *Am. J. Physiol.* **267,** G676–G686
40. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021
41. Schumacher, R., Soos, M. A., Schlessinger, J., Brandenburg, D., Siddle, K., and Ullrich, A. (1993) *J. Biol. Chem.* **268,** 1087–1094
42. Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S. (1994) *Cell* **78,** 35–43
43. Chiu, M. I., Katz, H., and Berlin, V. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12574–12578
44. Sabers, C. J., Martin, M. M., Brunn, G. J., Williams, J. M., Dumont, F. J., Wiederrecht, G., and Abraham, R. T. (1995) *J. Biol. Chem.* **270,** 815–822
45. Cooper, J. A. (1991) *Methods Enzymol.* **201,** 251–261
46. Lin, T.-A., Kong, X., Saltiel, A. R., Blackshear, P. J., and Lawrence, J. C., Jr. (1995) *J. Biol. Chem.* **270,** 18531–18538
47. Lazar, D. F., Wiese, R. J., Brady, M. J., Mastick, C. C., Waters, S. B., Yamauchi, K., Pessin, J. E., Cuatrecasas, P., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270,** 20801–20807
48. Hunter, T., and Karin, M. (1992) *Cell* **70,** 375–387
49. Christy, R. J., Kaestner, K. H., Geiman, D. E., and Lane, M. D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2593–2597
50. Moxham, C. P., Duronio, V., and Jacobs, S. (1989) *J. Biol. Chem.* **264,** 13238–13244
51. Chou, M. M., and Blenis, J. (1995) *Curr. Opin. Cell Biol.* **7,** 806–814