Insulin inhibits the expression of the hepatic insulin-like growth factor-binding protein-1 (IGFBP-1) and glucose-6-phosphatase (G6Pase) genes. The signaling pathway that mediates these events requires the activation of phosphatidylinositol-3 kinase, whereas transfection studies have suggested an involvement of Akt (protein kinase B) and FKHR, a transcription factor regulated by Akt. We now demonstrate that insulin repression of endogenous IGFBP-1 gene transcription was blocked by rapamycin or by amino acid starvation. Rapamycin inhibited the mammalian target of rapamycin (mTOR) and the subsequent activation of p70/p85 S6 protein kinase-1 (S6K1) by insulin, whereas amino acid depletion prevented insulin induction of these signaling molecules. Importantly, we demonstrate that insulin regulation of the thymine-rich insulin response element of the IGFBP-1 promoter was also inhibited by rapamycin. However, sustained activation of S6K1 did not repress this promoter. In addition, rapamycin did not affect insulin regulation of G6Pase expression or Akt activation. We propose that these observations indicate that mTOR-dependent, but S6K-independent mechanism regulates the suppression of IGFBP-1 (but not G6Pase) gene expression by insulin. Therefore, although the insulin-responsive sequence of the G6Pase gene promoter is related to that of the IGFBP-1 promoter, the signaling pathways that mediate suppression of these genes are distinct.

Insulin regulates hepatic homeostasis by inducing a complex signaling network that ultimately mediates the ability of the liver to maintain glucose homeostasis (1–4). Defects in this signaling network may underlie the development of insulin resistance. Upon binding to its cognate receptor at the cell membrane, insulin promotes a conformational change that results in autophosphorylation and activation of the intracellular domain of the receptor. This activation of the insulin receptor tyrosine kinase promotes phosphorylation of a number of intracellular substrates, including a group of related proteins known as insulin receptor substrates 1–4 (5). These proteins act as adapters, recruiting and activating proteins that bind to the phosphotyrosine motifs targeted by the insulin receptor. One such molecule is the lipid kinase phosphatidylinositols 3-kinase (PI3K). Activation of PI3K results in the production of 3-phosphorylated inositides, including phosphatidylinositol 3,4,5-trisphosphate. These molecules interact with multiple pleckstrin homology domain-containing proteins, including the protein kinases 3-phosphoinositide-dependent protein kinase-1 and Akt (also known as protein kinase B) (6, 7). Accumulation of phosphatidylinositol 3,4,5-trisphosphate activates S6 protein kinase (S6K), Akt, and serum- and glucocorticoid-regulated protein kinase (SGK) through a 3-phosphoinositide-dependent protein kinase-1-dependent mechanism (for reviews, see Refs. 8 and 9). In addition, PI3K activation also regulates signaling through the protein kinase termed mammalian target of rapamycin (mTOR) by a less well defined mechanism that may require Akt activation (10, 11). Activation of S6K (but not Akt or SGK) requires both PI3K and mTOR activity (11–13).

In yeast, TOR signaling is known to regulate the cellular localization of specific transcription factors (14), whereas in mammals, the ciliary neurotrophic factor regulates STAT3 phosphorylation and activation is mediated via mTOR (15). Downstream of mTOR, S6K phosphorylates ribosomal S6 protein in vitro and in vivo (12, 13) and may be involved in insulin regulation of protein translation (16, 17). Mammals express two homologous S6K proteins from distinct genes, termed S6K1 and S6K2. Mice lacking S6K1 have a reduced pancreatic β-cell size and are thus hypoinsulinemic and glucose-intolerant (18).

The mTOR pathway has been linked to the regulation of expression of the insulin gene in pancreatic β-cells (19), the Na+/P, cotransporter-1 gene in H4IE cells (20), the hexokinase II gene in L6 myotubes (21), and the p85α-regulatory subunit of PI3K in isolated muscle cells (22). The molecular connection between mTOR and these gene promoters is not clear. In contrast, Akt and SGK have been implicated in the regulation of multiple genes through the phosphorylation of FKHR/AFX (23–28), related members of a subfamily of...
the FOX(o) transcription factor family (29). For example, overexpression studies and treatment of cells with selective PI3K inhibitors have implicated PI3K, Akt, and FKHR in the regulation of IGFBP-1 gene expression by insulin (25, 30–32). A well characterized thymine-rich insulin response element (TIRE) is fundamental to the correct regulation of the IGFBP-1 promoter by insulin (30, 33), and FKHR-related proteins can bind to this TIRE (T(G/A)TTT/G(T)/G) in vitro (34). Homologous TIRE sequences are found in a number of other insulin-repressed gene promoters, including the glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) promoters. Therefore, a PI3K-, Akt-, and FKHR-dependent mechanism for regulation of G6Pase and PEPCK expression has also been proposed (35).

However, some recent studies have questioned this hypothesis. For example, the mTOR inhibitor rapamycin blocks the regulation of glucocorticoid-induced IGFBP-1 expression by insulin (36). In addition, detailed characterization of the IGFBP-1 and PEPCK TIRE sequences suggests that FKHR can bind to mutant TIRE sequences that do not respond to insulin (37).

To resolve this discrepancy, we have carried out a detailed analysis of the effect of rapamycin on the regulation of basal and glucocorticoid-induced IGFBP-1 and G6Pase gene transcription as well as its effect on insulin regulation of the IGFBP-1 TIRE, with and without FKHR overexpression. We found that mTOR activity is required for full insulin regulation of IGFBP-1 (but not G6Pase or PEPCK) gene transcription and that S6K activation is not sufficient to reproduce this action of insulin.

**EXPERIMENTAL PROCEDURES**

**Materials**—The radiolabeled [γ-32P]ATP (Amersham Biosciences, Inc., Little Chalfont, Buckinghamshire, United Kingdom) and [α-32P]UTP (ICN, Thame, Oxfordshire, UK) was obtained from the indicated sources. Insulin was purchased from Novo Nordisk (Crawley, West Sussex, UK). 8-(4-Chlorophenylthio)-cAMP was from Roche Molecular Biologicals (Lewes, East Sussex, UK). Wortmannin, LY294002, and rapamycin were from Calbiochem. The RPII kit was from Intron Inc. (Austin, TX). Protein G-Sepharose conjugated to the appropriate antibody. The immunocomplexes were pelleted by centrifugation at 30,000 g at 4 °C overnight prior to incubation for 1 h at room temperature with secondary antibody and development using the ECL kit (Amersham Biosciences, Inc.) following the manufacturer’s instructions. 4-Hydroxytamoxifen (Sigma) following the manufacturer’s instructions, with 5% polyacrylamide gel; band intensity was quantitated on a PhosphorImager (Fuji); and data were calculated as the ratio of IGFBP-1 or G6Pase to β-actin RNA. An RNase protection assay was carried out to determine the relative amounts of IGFBP-1, G6Pase, β-actin, or cyclophilin mRNA in each sample. The synthesis of the rat IGFBP-1 and G6Pase cDNA has been described previously (38, 39). A similar strategy was used to construct mouse IGFBP-1 and G6Pase probes. Briefly, mouse cDNAs for IGFBP-1 (from nucleotide +625 to +830) or G6Pase (from nucleotide +63 to +237) were amplified from a mouse liver cDNA library (CLONTECH), and the resultant product was cloned into pCR4-TOPO (Invitrogen). The plasmid was cut with SpeI to produce a linear DNA template. pTRI-cyclophilin (rat) and pTRI-actin (mouse) linear plasmids (Ambion Inc.) were used as control DNA templates. All RNA probes were synthesized by in vitro transcription following the instructions of the MaxiScript kit (Ambion Inc.). 3000 cpm of each probe was hybridized with 10 μg of total RNA following the instructions of the MaxiScript kit (Ambion Inc.). Cell membranes were removed by centrifugation at 13,000 x g for 5 min, and the protein concentration was determined by the method of Bradford (65) using bovine serum albumin as an internal standard.

**Antibodies for Immunoprecipitation and Western Blot Analysis**—The phospho-specific Ser-235 ribosomal S6 protein antibody was raised against a peptide derived from amino acids 229–242 (AKRRRLpSSL-RASTS) and is commercially available from Upstate Biotechnology, Inc. (TIB): The phospho-specific Thr-202/Tyr-204 p42/p44 MAPK antibody was purchased from New England Biolabs, Inc. Immunoprecipitation of endogenous FKHR was performed as described above, and total or isolated protein was separated on Novex SDS-4–12% polyacrylamide gel. Following transfer to nitrocellulose, blots were incubated with 5% (v/v) nonfat milk for 2 h, and primary antibodies were incubated at 4 °C overnight prior to incubation for 1 h at room temperature with secondary antibody and development using the ECL kit (Amersham Biosciences, Inc.) following the manufacturer’s instructions. 1 unit of kinase activity is the amount that catalyzes the phosphorylation of 1 nmol of substrate in 1 min. Antigens with the phospho-specific Ser-235 ribosomal S6 protein antibody was raised against a peptide derived from amino acids 229–242 (AKRRRLpSSL-RASTS) and is commercially available from Upstate Biotechnology, Inc. (TIB): The phospho-specific Thr-202/Tyr-204 p42/p44 MAPK antibody was purchased from New England Biolabs, Inc. Immunoprecipitation of endogenous FKHR was performed as described above, and total or isolated protein was separated on Novex SDS-4–12% polyacrylamide gel. Following transfer to nitrocellulose, blots were incubated with 5% (v/v) nonfat milk for 2 h, and primary antibodies were incubated at 4 °C overnight prior to incubation for 1 h at room temperature with secondary antibody and development using the ECL kit (Amersham Biosciences, Inc.) following the manufacturer’s instructions. 1 unit of kinase activity is the amount that catalyzes the phosphorylation of 1 nmol of substrate in 1 min.

**Plasmids and Transient Transfections**—The plasmids BP-1WT and BP-1DM5 were a gift from Dr. Rob Hall and Professor Daryl K. Granner (Vanderbilt University, Nashville, TN). The BP-1WT plasmid represents a luciferase reporter construct under the control of a thymidine kinase promoter containing the IGFBP-1 wild-type TIRE sequence (5’-CAAAACAAACTTATTTTG). Two base pair mutations of the wild-type TIRE sequence at residues equivalent to position 5 in each of the A and B sites (5’-CAAAAGAAACTTCTTTTG) produces a mutant promoter (BP-1DM5) that is no longer responsive to insulin (37). Transfections were performed using the calcium phosphate method as described previously (42). H4IIE cells were transfected with 10 μg of BP-1WT or BP-1DM5 with or without 10 μg of glutathione S-transferase-FKHR (25). Cells were then incubated for 20 h in serum-free medium with or without inhibitors as described in the figure legends. Cells were lysed in 900 μl of L cell lysis buffer (Promega, Southampton, UK) and centrifuged at 13,000 rpm for 2 min, and the supernatant was stored at −70 °C. Luciferase assays were performed using the firefly luciferase assay system (Promega) following the manufacturer’s instructions, with the luciferase activity being corrected for the protein concentration in the cell lysate as determined by the method of Bradford (65).

**Isolation of Nuclei and Nuclear Run-on Assay**—Nuclei from H4IIE cells were isolated by a modification of the method described previously (43). Briefly, cells were harvested in ice-cold phosphate-buffered saline, pelleted at 1200 rpm for 5 min, and resuspended in 4 ml of sucrose buffer I (0.3 M sucrose, 3 mM CaCl2, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl [pH 8], 1 mM dithiothreitol, and 0.5% (v/v) Nonidet P-40). The mixture was then transferred to a Wheaton Dounce tissue grinder, and the cells were lysed by five strokes each with a type B pestle followed by a type A pestle. 4 ml of sucrose buffer II (2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl [pH 8], and 1 mM dithiothreitol) was added to the homogenate. Nuclei were collected by overlying 4.4 ml of sucrose buffer II with the sample and ultracentrifugation at 30,000 × g for 45 min at 4 °C. The nuclei pellet was suspended in 300 μl of sucrose buffer II (50 mM Tris-HCl [pH 8.3], 40% (v/v) glycerol, 5 mM MgCl2, and 0.1 mM EDTA) and stored at −80 °C. A cDNA for rat IGFBP-1 (see “RNA Isolation and RNase Protection Assay”), a positive control cDNA (rat β-actin, and a negative control cDNA (pCR2.1, Invitrogen) were linearized, denatured in 0.3 M NaOH; applied (5 μg/μl) to 0.45-μm nitrocellulose membrane; and fixed by UV...
cross-linking. The rat β-actin was amplified from total rat liver mRNA by reverse transcription-PCR using primer sequences 5′-TCA TGA AGT GTG ACG TTG ACA TCC GT-3′ and 5′-CCT AGA AGG ATT TGC GGT GCA CGA TG-3′ (Promega). The resultant product was cloned into pCR2.1 and linearized with BamHI. The negative clone (pCR2.1) was created by digesting pCR2.1 with EcoRI to remove the β-actin insert and religating the vector to produce the empty vector. To examine transcription initiation, isolated nuclei (∼50 × 10⁶) were incubated in 5 mM Tris-HCl (pH 8), 2.5 mM MgCl₂, 150 mM KCl, 0.25 mM ATP, 0.25 mM CTP, 0.25 mM GTP, and 150 μCi of [α-³²P]UTP for 30 min at 37 °C. Proteins and DNA were removed by phenol/chloroform extraction. The radiolabeled RNA was recovered by ethanol precipitation, and the total incorporation of radioactivity for each RNA sample was determined by scintillation counting (44). Equal amounts of radiolabeled RNA (6 × 10⁶ cpm) were incubated with the nitrocellulose-immobilized DNA slot blots in a final volume of 1 ml of TES/NaCl solution (10 mM TES (pH 7.4), 10 mM EDTA, 0.2% (v/v) SDS, and 0.6 mM NaCl) for at least 36 h. After hybridization, the membranes were washed with 2× SSC at 65 °C for 1 h, followed by 2× SSC containing 0.1% SDS at 65 °C for 1 h. An additional wash was performed in the presence of 10 μg/ml RNase A at 37 °C for 30 min to digest any radiolabeled RNA that had not annealed to the membrane. The RNase was removed by a single wash with 2× SSC for 1 h. The blots were visualized by autoradiography and PhosphorImager analysis.

Preparation of Adenoviruses and Treatment of H4IIE Cells with Adenoviruses—Adenoviral vectors were generated using the system described by Hardy et al. (45). Briefly, an XbaI-PstI fragment containing the S6K1 cDNA (either wild-type or kinase-dead due to mutation to amino acids 552 and 553) was subcloned into pAdlox cut with XbaI and PstI. 10 μg of the resulting plasmids was cotransfected together with 3 μg of the donor viral DNA 75 in 293 cells expressing the recombinase CRE (CRES cells). When cells rounded up, they were collected, and an extract was prepared by sonication. The extracts were used for further amplification of the adenoviral vectors in CRES cells. All additional viral amplifications were carried out in human embryonic kidney 293 cells. H4IIE cells were infected at a multiplicity of infection of 5 in serum-free DMEM. After 36 h, cells were treated with or without insulin and lysed (as described above) for Western blot analyses, or RNA was prepared for gene expression analysis.

Statistical Analyses—As a measure of statistical significance of differences in experimental groups, Student’s t tests were performed, and 5% confidence limits were applied.

RESULTS

The regulation of basal or glucocorticoid/cAMP-induced G6Pase gene expression in H4IIE cells by insulin was blocked by inhibitors of PI3K, but was not affected by the presence of rapamycin (Fig. 1, A and B). In contrast, inhibition of mTOR by treatment of H4IIE cells with as little as 1 nM rapamycin strongly antagonized the ability of insulin to repress either basal (Fig. 1C) or glucocorticoid-induced (Fig. 1D) IGFBP-1 gene expression. However, this action of insulin was not completely inhibited by this treatment, even at rapamycin concentration as high as 200 nM (data not shown). Consistent with these findings, nuclear run-on analysis demonstrated that this mTOR-dependent pathway mediated about half of the insulin regulation of IGFBP-1 gene transcription (Fig. 2).

Transient transfection of H4IIE cells with a luciferase reporter gene (BP-1WT) under the control of a thymidine kinase promoter containing the IGFBP-1 TIRE (nucleotides –96 to –72 relative to the transcription start site) rendered luciferase expression sensitive to insulin (Fig. 3). The response of this TIRE to insulin was ablated by two base pair mutations (BP-1DM5) of residues equivalent to position 5 in each of the A and B sites of the IGFBP-1 TIRE (Fig. 3) (37). Meanwhile, the ability of insulin to regulate BP-1WT was completely lost in the presence of 10 nM rapamycin (Fig. 3A). Previously, this element was proposed to be regulated independently of mTOR activity, via Akt regulation of FKHR (25, 30–32). We therefore checked whether FKHR is also regulated in a rapamycin-sensitive manner. When FKHR was coexpressed with BP-1WT in H4IIE cells, the expression of luciferase was induced by >5-fold, whereas insulin produced a 50% block of this induced expression (Fig. 3B). Interestingly, the ability of insulin to inhibit this FKHR-mediated luciferase expression in H4IIE cells was slightly reduced in the presence of 10 nM rapamycin (Fig. 3B); however, the effect of rapamycin on FKHR activity was not as significant as the rapamycin block of insulin regulation in the absence of FKHR overexpression (Fig. 3, A and B). Coexpression of FKHR with BP-1DM5 in H4IIE cells did not induce luciferase expression or impart insulin sensitivity to this promoter (Fig. 3B), consistent with its inability to bind to this mutant TIRE (37).

Importantly, treatment of H4IIE cells with as low as 1 nM rapamycin completely blocked the activation of S6K1 by insulin (Fig. 4A). However, the presence of 10 nM rapamycin did not affect the ability of insulin to activate MAPK (Fig. 4B) or to induce phosphorylation of the Akt substrates GSK3α and GSK3β at Ser-21 and Ser-9, respectively (Fig. 4B), or FKHR at Thr-24, Ser-256, and Ser-319 (Fig. 4C). Insulin did not activate SGK in H4IIE cells (data not shown).

Depriving cells of amino acids is an alternative mechanism for inactivating the mTOR/S6K pathway (47, 48). The ability of insulin to induce phosphorylation of S6 in H4IIE cells deprived of amino acids and glucose for 1 h was almost completely lost (Fig. 5A). Meanwhile, Akt activation by insulin was not affected by amino acid deprivation, but basal Akt activity was

**Fig. 1.** Insulin regulation of IGFBP-1 (but not G6Pase) gene expression is rapamycin-sensitive. H4IIE cells were serum-starved overnight and preincubated for 30 min with or without inhibitors or Me₂SO carrier as indicated. The cells were then subjected to a 3-h incubation with hormones ± rapamycin, wortmannin, and LY294002 (wortmannin, 100 nM; LY294002, 100 μM; dexamethasone, 500 nM; 8-(4-chlorophenylthio)-cAMP; 0.1 mM; and insulin, 10 nM). Total cellular RNA was isolated, and an RNase protection assay was performed to assess the levels of G6Pase (A and B) and IGFBP-1 (BP-1) (C and D). Each experiment was performed at least twice in duplicate. Representative experiments (A–D) as well as quantification of four experiments (upper panels) are shown for IGFBP-1 analyses (C and D). Results are means ± S.E., presented either as percentage gene expression relative to control (C) or -fold induction over control (D). *** p < 0.001.
The Insulin Signaling Pathway That Regulates IGFBP-1 Gene Expression Is Distinct from the Pathway That Regulates G6Pase and PEPCK Gene Expression

The search for insulin-responsive DNA promoter elements and their binding proteins has led to the identification of a TIRE in the PEPCK promoter and subsequently to the identification of similar insulin-responsive sequences within a number of other gene promoters, including IGFBP-1 and G6Pase (35, 49, 50). Recently, FKHR and related transcription factors have been shown to bind to TIREs in vitro and to regulate their activity in intact cells (25, 30–32, 34). The discovery that FKHR is regulated in a PI3K- and Akt-dependent manner suggests a potentially complete signaling pathway from the insulin receptor to a subgroup of insulin-regulated gene promoters. However, we now demonstrate that complete repression of the IGFBP-1 TIRE requires mTOR activity, whereas G6Pase or PEPCK (51) expression is insensitive to rapamycin. Thus, it seems that these related DNA sequences are actually regulated by distinct mechanisms. Indeed, although there is a great deal of circumstantial evidence linking Akt and FKHR to regulation of IGFBP-1, G6Pase, and PEPCK expression, there are also a number of reports suggesting that the regulation of G6Pase and PEPCK expression by insulin may require other signaling pathways. For example, Tavare and co-workers (52) were unable to repress the G6Pase promoter by cotransfection of an active form of Akt. Although these workers did not check FKHR activity in their experiments, others have demonstrated strong repression of FKHR when Akt is activated. Similarly, Granner and co-workers (37) have questioned the role of FKHR as a regulator of the PEPCK and IGFBP-1 promoters. A base-by-base examination of the PEPCK (and IGFBP-1) TIRE revealed that FKHR-L1 could bind to mutant TIRE sequences that had lost the ability to mediate a response to insulin. Also, overexpression of “dominant-negative” Akt reduces the regulation of
FKHR by insulin, but not the regulation of the endogenous PEPCK promoter (53). Although it has been shown that overexpression of active Akt reduces PEPCK expression, this effect can be explained by inhibition of GSK3 (a physiological target of Akt) (40). Inhibitors of GSK3 strongly reduce PEPCK and G6Pase gene transcription in H4IIE cells (54). The data presented herein demonstrate that the regulation of IGFBP-1 gene transcription by insulin is affected by the presence of rapamycin, a treatment that does not affect insulin regulation of G6Pase (Fig. 1) or PEPCK (51). Thus, mTOR is an important mediator of insulin regulation of the IGFBP-1 promoter. These results demonstrate that distinct mechanisms regulate these three TIREs in vivo.

The IGFBP-1 Promoter May Contain an Element, Other than the TIRE, That Enhances Insulin Repression of Endogenous IGFBP-1 Gene Transcription—Surprisingly, the regulation of the IGFBP-1 TIRE by insulin is completely blocked by rapamycin, whereas endogenous gene regulation is only affected partially (~50%). This suggests that a second insulin response element, or an accessory factor element, may be present within the IGFBP-1 promoter and that this second element is rapamycin-insensitive. Indeed, heterologous promoters containing...
The Role of mTOR, S6K, and FKHR in the Regulation of IGFBP-1 Gene Expression—The effects of rapamycin (Fig. 2) and amino acid deprivation (Fig. 5) demonstrate a requirement for mTOR activity in the complete repression of IGFBP-1 gene transcription by insulin. However, S6K activity is not sufficient to replace insulin regulation of this gene (Fig. 6). Initial experiments in mice lacking both the S6K1 and S6K2 genes suggest that the regulation of IGFBP-1 expression is not affected by loss of S6K activity (data not shown). This suggests either that S6K is not required for the regulation of this gene by insulin or that these animals have adapted by up-regulating an alternative activity. This requires more detailed analysis, but it remains a distinct possibility that S6K is neither sufficient nor required for the regulation of IGFBP-1 expression by insulin.

In yeast, Tor signaling produces cytoplasmic sequestration of a number of transcription factors known to mediate a response to nutrients (14, 56, 57). Specifically, starved-down-regulates Tor activity and induces nuclear translocation of factors such as Msn2p, Msn4p, and Gln3p (14, 58). Tor has also been shown to regulate the expression of metabolic genes via the factors Tap42p, Mks1p, Ure2p, Gln3p, and Gat1p (57, 59). Indeed, rapamycin treatment of yeast for as little as 30 min has profound effects on gene expression, both positively and negatively (57). However, yeast cells do not express an S6K homolog; and thus, the mechanism by which Tor regulates these transcription factors must involve a distinct Tor signaling cascade (11). For example, Tap42p is a phosphatase-associated protein that can interact with mammalian protein phosphatases 2A and 4 (60, 61). It is therefore tempting to speculate that mTOR may regulate the mammalian transcriptional machinery through activation of a phosphatase. Meanwhile, the inhibition of mTOR leads to the induction of autophagy in hepatocytes, whereas persistent activation of S6K inhibits autophagy (48, 62). The effects that we observed on IGFBP-1 gene transcription in the presence of rapamycin cannot therefore be linked to increased autophagy.

Previously, there was no evidence that an mTOR-activated pathway could regulate FKHR activity. The serine and threonine residues targeted by Akt (and/or SGK) have been well characterized, and mutation of these residues to alanine (AAA-FKHR) renders FKHR almost totally insensitive to insulin (32, 37). We have demonstrated that Akt-mediated phosphorylation of FKHR occurs in rapamycin-treated H4IIE cells (Fig. 4). It is difficult to assess the effect of rapamycin on insulin regulation of FKHR transactivating activity due to an inductive effect of rapamycin in the absence of insulin (Fig. 3B). In the presence of insulin, rapamycin returns promoter activity to the baseline level, but not to the rapamycin-induced level. Indeed, there is not a significant difference in the effect of insulin when FKHR is overexpressed in the presence or absence of rapamycin (p > 0.1), although there is a trend toward a small effect of rapamycin on FKHR transactivating potential. This suggests the existence of an FKHR-independent regulator of the IGFBP-1 TIRE, unless overexpression of FKHR renders it less sensitive to rapamycin. It will require a much more detailed study of endogenous FKHR phosphorylation and activity to determine the relative contributions of these insulin signaling pathways to the regulation of FKHR. Taken together with previous results, it has now been established that insulin-mediated regulation of promoters is profoundly altered following overexpression of FKHR/FKHR-L1. An important issue in this field is to establish whether FKHR and FKHR-L1 are the true regulators of TIREs or whether overexpression alters the TIRE-binding complex. The generation of FKHR/FKHR-L1/AFX-deficient animals may be required to identify the bona fide target gene promoters for these factors.
In conclusion, although distinct regulation of the IGFBP-1 and G6Pase gene promoters by phorbol esters has been demonstrated previously (39), this work is the first demonstration of a difference in the specific insulin signaling molecules required for repression of the G6Pase and IGFBP-1 genes. The requirement for mTOR (but not S6K) activity is analogous to Tor regulation of gene transcription in yeast. In addition, we propose the existence of a second insulin response element within the IGFBP-1 promoter that is insensitive to rapamycin and suggest that the transcription factor FKHR may not be the endogenous regulator of the IGFBP-1 TIRE.

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