Regulation of Phosphoenolpyruvate Carboxykinase and Insulin-like Growth Factor-binding Protein-1 Gene Expression by Insulin

THE ROLE OF WINGED HELIX/FORKHEAD PROTEINS*

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Winged helix/forkhead (Fox) transcription factors have been implicated in the regulation of a number of insulin-responsive genes. The insulin response elements (IREs) of the phosphoenolpyruvate carboxykinase (PEPCK) and insulin-like growth factor-binding protein-1 (IGFBP-1) genes bind members of the FKHR and HNF3 subclasses of Fox proteins. Previous mutational analyses of the PEPCK and IGFBP-1 IREs revealed mutations which do not affect the binding of HNF3 proteins to these elements but do eliminate the ability of the IREs to mediate an insulin response. This dissociation of binding and function provided compelling evidence that HNF3 proteins, per se, are not insulin response proteins. The same approach was used here to determine if FKHRL1, a member of the FKHR subclass of Fox proteins, binds to the PEPCK and IGFBP-1 IREs in a manner that correlates with the ability of these elements to mediate an insulin response. Overexpression of FKHRL1 stimulates transcription from transfected reporter constructs that contain a multimerized PEPCK IRE or an IGFBP-1 IRE and this stimulation is repressed by insulin. There is a direct correlation between the ability of mutant versions of the PEPCK and IGFBP-1 IREs to bind FKHRL1 and their ability to mediate FKHRL1-induced transcription when FKHRL1 is overexpressed. However, under conditions where FKHRL1 is not overexpressed, there is a lack of correlation between FKHRL1 binding to mutant versions of the PEPCK and IGFBP-1 IREs and the ability of these elements to mediate an insulin response. Therefore, the PEPCK and IGFBP-1 IREs mediate FKHRL1-induced transcription and its inhibition by insulin when this protein is overexpressed, but at the normal cellular concentration of FKHRL1 the insulin response mediated by these elements must involve another protein.

Insulin affects the expression of over 100 genes (1, 2). Many of these genes are regulated by insulin at the transcriptional level, but the molecular details by which this regulation is achieved are poorly understood. Progress in this area has been hampered by the fact that there is no consensus insulin response element (IRE) that can account for the regulation of all insulin-responsive genes. However, an IRE with a T(G/A)T[T/G]/G/T core sequence has been associated with insulin-induced transcriptional repression of a number of metabolic genes, including those that encode phosphoenolpyruvate carboxykinase (PEPCK), insulin-like growth factor-binding protein-1 (IGFBP-1), tyrosine aminotransferase, glucose-6-phosphatase, apolipoprotein C III, and aspartate aminotransferase (3–8). Trans-acting factors that interact with the IREs of these genes have been identified, but none have been directly shown to mediate an insulin response.

The PEPCK IRE co-localizes with an element in the PEPCK promoter termed the glucocorticoid accessory factor 2 (gAF2) element (3, 9). The gAF2 element is a component of the PEPCK glucocorticoid response unit and is required for a full glucocorticoid response by the PEPCK gene (10, 11). Winged helix/forkhead (Fox) proteins of the HNF3 subclass are responsible for the accessory activity of gAF2 (12, 13). The co-localization of the IRE with gAF2 led to the hypothesis that HNF3 proteins might also be involved in mediating the insulin response (2, 12). However, point mutation analysis of the gAF2 element showed that there is no correlation between the binding of HNF3 proteins and the ability of the element to confer insulin responsiveness to a heterologous promoter (13).

The IGFBP-1 IRE, like the PEPCK IRE, is involved in the regulation of IGFBP-1 gene expression by glucocorticoids, and HNF3 proteins mediate this function (12, 14–16). However, mutant versions of the IGFBP-1 IRE, which bind HNF3 proteins but cannot mediate an insulin response, have also been described (16). Thus, HNF3 proteins are not solely responsible for an insulin response mediated by the PEPCK and IGFBP-1 IREs.

Members of the FKHR subclass of the Fox family of transcription factors, namely FKHR, AF2, and FKHRL1, may be involved in the regulation of PEPCK and IGFBP-1 gene expression by insulin (17–22). Interest in FKHR and related proteins arose from the finding that an insulin-like signaling pathway exists in the nematode Caenorhabditis elegans (23–29). This pathway consists of DAF-2, AGE-1, PDK-1, and AKT 1, 2 which are the C. elegans homologs of the insulin receptor, the catalytic subunit of PI 3-kinase, the 3-phosphoinositide-dependent

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1 The abbreviations used are: IRE, insulin response element; PEPCK, phosphoenolpyruvate carboxykinase; IGFBP-1, insulin-like growth factor-binding protein-1; PKB, protein kinase B; GST, glutathione S-transferase; wt, wild type; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; luc, luciferase; PI, phosphatidylinositol; FKHR, gAF2, glucocorticoid accessory factor 2.
kinase-1, and protein kinase B (PKB), respectively. Genetic complementation experiments showed that activation of this pathway leads to the inactivation of DAF-16, the *C. elegans* homolog of FKHR. The subsequent identification of DAF-16 as a Fox family member led to the speculation that FKHR proteins might be downstream targets of insulin-activated PKB in mammalian cells and might be responsible for mediating the regulation of gene expression by insulin. Indeed, the FKHR proteins are phosphorylated by PKB in vitro and in vivo, and FKHR proteins can bind the PEPCCK and IGFBP-1 IREs (17, 18, 21, 22, 30). In the case of FKHR1, the protein used in this study, activated PKB phosphorylates Thr32 and Ser253 in vivo, but a third site, Ser315, appears to be phosphorylated by a different kinase. Phosphorylation of Thr32 and Ser253 in FKHR1 leads to the sequestration of the protein in the cytoplasm, thus removing it from its site of action in the nucleus (21).

Several studies have shown that the overexpression of FKHR proteins in cells leads to a stimulation of transiently transfected reporter constructs containing the IGFBP-1 IRE, and that this transactivation is blocked by treatment of the transfected cells with insulin (17, 19, 20). However, high level expression of FKHR proteins in cells could mask the existence of another protein that mediates the insulin response under physiological conditions. Therefore, in the present study, wild-type and mutant versions of the PEPCCK and IGFBP-1 IREs were analyzed for their ability to confer insulin responsiveness to a heterologous promoter in the presence or absence of overexpressed FKHR1.

There is a direct correlation between the binding of FKHR1 to the PEPCCK and IGFBP-1 IREs and the ability of these elements to mediate transactivation when FKHR1 is overexpressed. Insulin represses the transcriptional activation caused by overexpressed FKHR1. However, the insulin response mediated by the PEPCCK and IGFBP-1 IREs in the absence of overexpressed FKHR1 does not correlate with FKHR1 binding. We suggest that another factor must be required for the PEPCCK and IGFBP-1 IRE-mediated insulin response when FLKHK1 is at physiological levels in the cell.

**Experimental Procedures**

**Cell Culture and Transfection**—H4IIE rat hepatoma cells were maintained in α-minimum essential medium supplemented with 10% (v/v) serum (2% newborn, 0% calf, 5% fetal bovine). Transfections were performed using the calcium phosphate procedure as described previously (9), except that the cells were returned to α-minimum essential medium (10% serum) after exposure to the DNA precipitate. The cells were transfected with 10 μg of reporter plasmid and, where indicated, with 2.5 μg of FKHR1 expression vector or empty vector (PECE). The transfected cells were incubated 18 h in Dulbecco’s modified Eagle’s medium without serum and in the absence or presence of 10 nM insulin.

**CAT and Luciferase Assays**—CAT assays were performed according to the method of Nordeen (31) with minor modifications (32). Luciferase assays were performed using the Firefly luciferase assay system (Promega).

**Plasmid Construction**—The plasmid TKCVI contains the herpes simplex virus thymidine kinase (TK) promoter ligated upstream of the CAT gene (33). Double-stranded oligonucleotides, containing either the wt PEPCCK IRE sequence or a mutant version of the sequence, were cloned into the BamHI site of TKCVI to produce the PC42 series of plasmids (3). A PouIII-BglII fragment from PC42wt, PC42m5, or PC42m7 was cloned into SmaI-BglII cut pGL3-Basic (Promega) to construct the PL42 series of plasmids, which contain a luciferase (luc) reporter gene. The construction of the IG-IRE/TK series of plasmids began with the construction of TKLuc. This plasmid was derived from PL42wt by two site-directed mutagenesis steps, which created a single BamHI site at position −40 of the TK promoter in the context of pGL3. A BamHI site in the pGL3 vector was first eliminated by a single base change, and then a BamHI site was created upstream of the PEPCCK IRE insert in PL42wt. The resulting construct was cut with BamHI to remove the IRE insert, and this was then religated to create TKLuc. Double-stranded oligonucleotides containing a wt or mutant version of the IGFBP1 IRE were cloned into the BamHI site of TKluc to generate the IG-IRE/TK plasmid series. The IG-IRE/TK plasmids were then cloned into a double-stranded vector containing three copies of the PEPCCK IRE separated by XhoI restriction sites into XhoI-BglII cut pGL3-promoter vector (Promega). The plasmids pECE, pFKHRL1wt, and pFKHRL1tm (which contains alanines substituted for Thr22, Ser253, and Ser315) have been described elsewhere (21) and were provided by Anne Brunet and Michael Greenberg.

**Western Blots**—Whole cell lysates were subjected to electrophoresis on a 4–12% Bis-Tris gel (Novex) at 200 V for 1 h. The protein was transferred to a 0.45-μm nitrocellulose membrane (Novex) for 75 min at 30 V. After thorough washing, the membrane was treated with the primary antibody for 1 h at room temperature and then kept at 4 °C overnight. Alkaline phosphatase-conjugated, anti-rabbit secondary antibody treatment was added for 30 min, and a chemiluminescence assay was performed using the Western Breeze kit (Novex). Polyclonal anti-FKHRL1, anti-phospho-FKHRL1(Thr32), and anti-phospho-FKHRL1(Ser253) were obtained from Upstate Biotechnology.

**Electrophoretic Mobility Shift Assay (EMSA)**—Human FKHR1 cDNA cloned into pGEX 4t3 (Amersham Pharmacia Biotech) was a gift from Anne Brunet and Michael Greenberg. GST/FKHR was purified from Escherichia coli (BL21) using a glutathione-Sepharose 4B affinity column (Amersham Pharmacia Biotech). Conditions for EMSA were identical to those used for GST/FKHR (20). Briefly, 10 ng of GST/FKHR1 was incubated in a reaction mix containing 20 nM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol (v/v), 2 mM dithiothreitol, 2 mg/ml bovine serum albumin, 10 ng of poly(dG-dC), and ~5 fmol of radiolabeled probe. Unlabeled, competitor oligonucleotides were added before the addition of protein. The mixture was incubated at 4 °C for 15 min. Electrophoresis was carried out at 4 °C on a 5% polyacrylamide gel in 0.5× TBE running buffer.

**RESULTS**

**FKHR1 Phosphorylation in H4IIE Cells**—The presence of FKHR1 in hepatoma cells was determined by Western blot analysis using an FKHR1 antibody. FKHR1 migrates as a 100-kDa protein on an SDS gel and H4IIE and HepG2 cell extracts contain a 100-kDa protein that interacts with FKHR1 antibody (Fig. 1A). The difference in the intensity of the bands is presumably not due to a change in the amount of the protein, since a 15-min insulin treatment period was employed. The changes noted are more likely owing to an insulin-induced change in the epitope-antibody interaction (see below).

Residues Ser253 and Thr32 of FKHR1 are known targets of PKB-stimulated phosphorylation (21). The treatment of H4IIE cells with insulin results in a concentration-dependent phosphorylation of both sites as detected by phospho-Ser253 and phospho-Thr32 antibodies (Fig. 1, B and C). This phosphorylation is dependent on PI 3-kinase as the treatment of H4IIE cells in the presence of the PI 3-kinase inhibitor LY294002 blocked the insulin-induced phosphorylation (Fig. 1D). These data show that H4IIE cells contain FKHR1 and that this protein is phosphorylated in a manner consistent with that demonstrated in other cell types.

**Functional Analysis of the PEPCCK IRE**—Mutations of the PEPCCK IRE that affect binding of FKHR1 should also affect the ability of FKHR1 to transactivate and mediate an insulin response through this element. Transversion mutations were produced throughout the 10-base pair region that constitutes the PEPCCK IRE to address this hypothesis. The wt and mutant versions of the PEPCCK IRE were inserted 12 base pairs upstream of the TATA box in the TKCVI reporter vector to produce the PC42 family of plasmids (see Ref. 3 and “Experimental Procedures”). H4IIE cells were transfected with the series of plasmids, and the ability of insulin to repress transcription from the TK promoter was determined by the measurement of CAT activity (Fig. 2). A single copy of the wt PEPCCK IRE confers insulin responsiveness to the TK promoter such that insulin treatment of PC42wt-transfected cells causes about a 50% reduction of CAT activity as compared with untreated
cells. A triple transversion mutation of base pairs 1–3 of the IRE insert has no effect on the ability of the IRE to mediate an insulin response, nor did mutations at positions 4 or 8–10. Mutations of the central core at base pairs 5–7 had a significant effect on the ability of the IRE to mediate an insulin response in the context of TKCVI, and mutation of base pairs 5 or 7 completely abolished insulin responsiveness (Fig. 2).

Binding of FKHRL1 to the PEPCK IRE—The binding of partially purified GST/FKHRL1 was investigated by EMSA (see "Experimental Procedures"). A radiolabeled, double-stranded oligonucleotide that contains the PEPCK IRE was used as a probe and the competitor oligonucleotides contained either the wild-type IRE (wt), an IRE mutated at position 5 (m5), or an IRE mutated at position 7 (m7). The wt and m7 oligonucleotides competed equally well with the wt probe for binding of GST/FKHRL1 whereas the m5 oligonucleotide competed poorly (Fig. 3). Identical results were obtained when the binding of HNF3 proteins to the PEPCK IRE was examined (12, 13), which indicates that members of the Fox family of transcription factors may recognize this sequence in a similar manner. The ability of GST/FKHRL1 to bind to the m7-IRE as efficiently as it does to the wt-IRE indicates a dissociation of binding and function since the m7 mutation abolishes the ability of the IRE to mediate an insulin response (see Fig. 2).
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Functional Properties of FKHRL1 on the PEPCK IRE—The dissociation of binding from function noted above (compare Figs. 2 and 3) suggests that endogenous FKHRL1 per se is not involved in the physiologic regulation of the PEPCK gene IRE. Numerous studies, however, suggest that FKHR proteins, when overexpressed, are transactivators whose activity is repressed by insulin. Therefore, the ability of overexpressed FKHRL1 to transactivate and mediate an insulin response through various PEPCK IRE constructs was examined. Cotransfection of a FKHRL1 expression vector (pFKHRL1wt) with PL42wt has no effect on luciferase gene expression from PL42wt (Fig. 4B). Insulin represses the activity of PL42wt whether FKHRL1 is overexpressed or not. By contrast, the PL42m5 and PL42m7 constructs are unresponsive to insulin in either circumstance (Fig. 4, C and D). Therefore, overexpression of FKHRL1 cannot restore insulin responsiveness to PL42m5 or PL42m7 even though FKHRL1 binds to the m7-IRE as well as it binds to the wt-IRE (see Fig. 3). A triple mutant form of FKHRL1, FKHRL1tm, which has an alanine in the place of the PKB phosphorylation sites at Thr32, Ser253, and Ser315, was also tested for an effect on the PL42 reporter plasmids. This protein has no effect on the activity of PL42wt, PL42m5, and PL42m7 in the absence or presence of insulin (Fig. 4, B–D).

It is possible that the single PEPCk IRE in the PL42wt luciferase gene expression vector is not sufficient to mediate FKHRL1 activity in this context. Therefore, another set of luciferase reporter constructs (designated 3×PEP-IRE/SV, 3×PEP-IRE/SV(m5), and 3×PEP-IRE/SV(m7)) were generated. These all contain three tandem copies of the wild-type or mutant versions of the PEPCk IRE positioned upstream of the SV40 minimal promoter. Insulin represses basal reporter gene expression directed by the 3×PEP-IRE/SV construct. Overexpression of FKHRL1 causes a stimulation of 3×PEP-IRE/SV activity (Fig. 5B), and this stimulation is blocked by treatment of the cells with insulin. The overexpression of FKHRL1tm also stimulates 3×PEP-IRE/SV activity, but this stimulation is not prevented by insulin, thus indicating the importance of the PKB phosphorylation sites.

The m5-IRE in the 3×PEP-IRE/SV context is unable to mediate an insulin response and does not mediate FKHRL1-induced transactivation (Fig. 5C). This is consistent with the inability of PC42m5 (Fig. 2) and PL42m5 (Fig. 4C) to respond to insulin or be transactivated by FKHRL1, and with the observation that FKHRL1 binds poorly to the m5-IRE (see Fig. 3). The m7-IRE retains the ability to mediate FKHRL1-induced transactivation and FKHRL1 binds the m7-IRE as well as it does the wt-IRE (Fig. 3), thus explaining the equivalent level of FKHRL1-mediated transactivation of 3×PEP-IRE/SV and 3×PEP-IRE/SV(m7). However, the fact that insulin only represses 3×PEP-IRE/SV(m7) in the presence of overexpressed FKHRL1 illustrates a dissociation between the binding of FKHRL1 and the ability of insulin to repress gene transcription through the wild-type PEPCK IRE.

Binding of FKHRL1 to the IGFBP-1 IRE—The same approach was used to determine if the IGFBP-1 IRE is capable of mediating an insulin response in a FKHRL1-independent fashion. Binding studies were performed using a radiolabeled, double-stranded oligonucleotide that contains the wild-type IGFBP-1 IRE sequence as a probe. Oligonucleotides containing either wild-type or mutant versions of the IGFBP-1 IRE were used as competitors. The positions of the mutations introduced into the IGFBP-1 IRE relative to the PEPCk IRE are shown in Fig. 6. Mutations were produced at positions 5, 7, and 10 of the A and B halves of the IGFBP-1 IRE. Both halves were mutated because each half-site can bind a molecule of FKHRL1 (Fig. 7A).

An oligonucleotide containing the dm10 mutation competed...
as well as an oligonucleotide containing the wt IGFBP-1 IRE for binding of GST/FKHRL1 to the probe (Fig. 7B). The dm5 oligonucleotide did not appreciably compete for GST/FKHRL1 binding, whereas the dm7 oligonucleotide competed better than the dm5 oligonucleotide, but not as well as the wt or dm10 oligonucleotides.

**Functional Properties of FKHRL1 on the IGFBP-1 IRE**—A set of TK/luc reporter vectors were constructed in which the various IGFBP-1 IRE oligonucleotides were inserted into the same position of the TK promoter used for the PEPCK IRE as described in previous experiments (see “Experimental Procedures”). The wild-type IGFBP-1 IRE conferred insulin responsiveness through the TK/luc vector (Fig. 8B). Cotransfection of this plasmid with pFKHRL1wt resulted in a 3–4-fold increase of IG-IRE/TK reporter gene activity, and this effect was significantly blunted by insulin. The fact that overexpression of FKHRL1 stimulates reporter gene expression from constructs containing the IGFBP-1 gene IRE, but not from constructs containing a single copy of the PEPCK gene IRE, probably reflects the multimeric nature of the IGFBP-1 IRE. The degree of repression by insulin was significantly reduced when FKHRL1tm was overexpressed; thus, the ability of insulin to block FKHRL1 stimulation is at least partially dependent upon the PKB phosphorylation sites in FKHRL1.

Mutation of the base pairs at position 5 of the A and B sites in the IGFBP-1 IRE resulted in a complete loss of responsiveness to both insulin and FKHRL1 (Fig. 8C). Thus, these bases are critical for both insulin action and FKHRL1 binding. The IG-IRE/TK(dm7) reporter exhibited a loss of insulin responsiveness in the absence of overexpressed FKHRL1tm. However, this construct is activated when FKHRL1wt is overexpressed (Fig. 8D). Therefore, the dm7 mutation, like the analogous m7 mutation in the PEPCK IRE, highlights a dissociation between binding of FKHRL1 and the ability of the IGFBP-1 IRE to mediate an insulin response.

The most pronounced dissociation of FKHRL1 binding and insulin repression is demonstrated by IG-IRE/TK(dm10) (Fig. 8E).
FKHRL1 binding and FKHRL1-induced transactivation when position 10 as well as it does to the wild-type IGFBP-1 IRE; however, there is no repression by insulin of IG-IRE/Tk(dm10) in the absence of overexpressed FKHRL1. Therefore, the dm7 and dm10 mutant versions of the IGFBP-1 IRE support FKHRL1 binding and FKHRL1-induced transactivation when it is overexpressed, but they have lost the ability to mediate an insulin response in the absence of overexpressed FKHRL1.

**DISCUSSION**

These studies show that insulin can repress gene transcription through the PEPCK and IGFBP-1 IREs in an FKHRL1-dependent and -independent manner. FKHRL1, when overexpressed, binds to the PEPCK and IGFBP-1 IREs and activates transcription. This induced transactivation is blocked by insulin, but the insulin response mediated by the PEPCK and IGFBP-1 IREs appears to be independent of wild-type levels of FKHRL1. This conclusion is based on the binding and functional data presented here, which show that certain mutations in the PEPCK and IGFBP-1 IREs that do not affect FKHRL1 binding but abolish the ability of the IREs to mediate an insulin response. Therefore, it is unlikely that FKHRL1 is the physiologically relevant insulin response protein for the PEPCK and IGFBP-1 genes.

The same mutation (m7) that, in past studies, eliminated HNF3 from consideration as an insulin response protein proved to be pivotal in the binding/function analysis of FKHRL1 on the PEPCK IRE (see Ref. 13). The PC42m7 and PL42m7 reporter constructs do not respond to insulin in transiently transfected H4IIE cells. However, binding studies show that FKHRL1 binds the m7 version of the PCPEK IRE as strongly as it binds the wt-IRE. The ability of this mutation to block the insulin response through the PCPEK IRE is not limited to a particular promoter context, since it also abolishes the insulin response of 3×PEP-IRE/SV(m7). The results of these studies with constructs that contain the PCPEK IRE, performed in cells with their normal level of FKHRL1, indicate a dissociation of FKHRL1 binding from insulin-induced transcriptional repression.

A different scenario emerges when FKHRL1 is overexpressed in transfected cells. In this case, 3×PEP-IRE/SV and 3×PEP-IRE/SV(m7) reporter gene expression is equally enhanced by overexpressed FKHRL1 and insulin represses this transactivation. This result is not surprising since, as mentioned above, FKHRL1 binds the PCPEK m7-IRE as well as it binds the PCPEK wt-IRE. Thus, a correlation exists between the ability of FKHRL1 to bind to wt and mutant versions of the PCPEK IRE and insulin-induced transcriptional repression when the protein is expressed in excess of the wild-type level.

The IGFBP-1 IRE mediates transactivation by FKHRL, AFX, and FKHRL1 (19–21, 34). Mutations in the IGFBP-1 IRE were produced to extend and bolster the results obtained with the PCPEK IRE. The mutations at positions 5 and 7 in each half of the IGFBP-1 IRE were studied because they correspond to the m5 and m7 mutations in the PCPEK IRE. The mutation at position 10 was studied because it is important for IGFBP-1 IRE function but not PCPEK IRE function (13, 19). There is a correlation between the level of luciferase gene expression from the IG-IRE/Tk(dm7) and IG-IRE/Tk(dm10) constructs and the binding of FKHRL1 to the dm7 and dm10 IREs when this transcription factor is overexpressed. As with the PCPEK IRE, insulin represses this transcription. However, even though FKHRL1 binds to the dm7 and dm10 mutant IREs, reporter genes containing these elements exhibited no response to insulin when transfected into cells expressing normal levels of FKHRL1. Only the IG-IRE/Tk(wt) reporter gene was repressed by insulin in the absence of overexpressed FKHRL1. Therefore, two mutant versions of the IGFBP-1 IRE that bind FKHRL1 cannot mediate an insulin response in the absence of overexpressed FKHRL1. This dissociation of FKHRL1 binding from insulin action through the IGFBP-1 IRE provides strong evidence that FKHRL1, and probably other FKHR proteins, are not the sole physiologic mediators of insulin-induced transcriptional repression.

PKB activation (e.g. by IGF-1 or insulin), or the expression of constitutively active forms of PKB, repress transactivation mediated by FKHR proteins (17–19, 21, 22, 34–38). The involvement of PKB in the regulation of FKHRL1 activity by insulin is supported by our observation that a triple mutant of FKHRL1 (FKHRL1tm) in which the consensus PKB phosphorylation sites (Thr32, Ser253, and Ser315) are mutated does not respond to insulin. At least one of these PKB phosphorylation sites must be intact for insulin to repress FKHRL1 transactivation (21). Notably, FKHRL1tm transactivates promoter/reporter constructs containing either multiple copies of the PCPEK IRE or a single copy of the IGFBP-1 IRE, thus indicating DNA binding and transactivation functions are not disrupted by the triple mutation.

The results of this and other studies show that FKHRL1 binds the PEPCK and IGFBP-1 IREs, activates transcription through these elements, and that this activation is blocked by insulin. However, these effects appear to depend on FKHRL1 in excess of the normal cellular concentration. When FKHRL1 is at wild-type levels, insulin repression of transcription occurs in an FKHRL1-independent manner.

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