Gene- and Activation-specific Mechanisms for Insulin Inhibition of Basal and Glucocorticoid-induced Insulin-like Growth Factor Binding Protein-1 and Phosphoenolpyruvate Carboxykinase Transcription

ROLES OF FORKHEAD AND INSULIN RESPONSE SEQUENCES*

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The insulin response sequence (IRS) of the phosphoenolpyruvate carboxykinase (PEPCK) promoter, located within the glucocorticoid response unit, was first characterized by its ability to mediate insulin inhibition when inserted into a thymidine kinase promoter. The IRSs of the PEPCK and insulin-like growth factor binding protein-1 (IGFBP-1) promoters have been proposed to contribute to regulation by glucocorticoids and insulin. Forkhead (FKHR) recognizes IRS sequences, is phosphorylated in response to insulin, and mediates insulin inhibition of basal IGFBP-1 transcription in an IRS-dependent manner. Here, we investigate the contributions of FKHR and IRSs to insulin inhibition of basal and glucocorticoid-induced transcription of PEPCK and IGFBP-1. Expression of T/S/S, in which three putative protein kinase B (PKB) sites in FKHR are mutated, reduced insulin inhibition of basal expression of IGFBP-1 but not PEPCK. Mutation of the IGFBP-1 IRSs abolished insulin inhibition in the presence of T/S/S. Mutation of the PEPCK IRS had no effect on insulin inhibition in the presence of T/S/S, indicating that insulin inhibits PEPCK transcription independently of the IRS or of the putative PKB phosphorylation sites in FKHR. Mutations in the IRS or FKHR had no effect on insulin inhibition of glucocorticoid-induced transcription of either the PEPCK or IGFBP-1 gene. Thus, insulin uses gene- and activation-specific mechanisms to regulate the basal and glucocorticoid-induced activity of these genes.

Phosphoenolpyruvate carboxykinase (PEPCK)1 and insulin-like growth factor binding protein-1 (IGFBP-1) contribute to glucose homeostasis. PEPCK catalyzes the rate-limiting and committed step in gluconeogenesis (1, 2). IGFBP-1 binds IGFs in the serum and reduces the amount accessible to cells (3, 4). The regulation of PEPCK and IGFBP-1 is similar and is primarily a result of regulating the rate of gene transcription (5–8), which is induced by glucocorticoids and inhibited by insulin (9–13).

Glucocorticoids exert their effects on transcription by binding an intracellular receptor, causing it to translocate to the nucleus and bind to glucocorticoid response elements (GREs) present in the promoters of target genes (14–16). However, to effectively mediate induction, the glucocorticoid receptor must interact with accessory factors bound to a variety of sites within complex glucocorticoid response units (GRUs). The GRUs of the PEPCK and IGFBP-1 promoters are very similar, each containing two GREs and multiple accessory factor binding sites (13, 17). In the PEPCK promoter, the binding sites for accessory factor 1 (AF1) and accessory factor 2 (AF2), which have been shown to bind COUP-TF/HNF4 and forkhead/winged helix proteins, respectively, lie immediately 5′ of the GREs, whereas a third accessory factor site (AF3), which binds COUP-TF, is located 3′ of these sites (18–21). All five sites, as well as the cAMP response element (22), are necessary for maximal glucocorticoid induction of the PEPCK gene (17, 23, 24). The GRU of the human IGFBP-1 promoter also contains two GREs as well as two accessory factor sites, one of which binds HNF1 and is located downstream of the 3′ GRE, whereas the other binds forkhead/winged helix proteins and is located immediately upstream of the 3′ GRE (13, 18, 25).

O’Brien et al. (26) reported that a sequence present in the AF2 site of the PEPCK GRU conferred insulin inhibition on a linked reporter when inserted into a heterologous thymidine kinase promoter (26). Mutation of this sequence abolished both insulin inhibition of the thymidine kinase promoter and the binding of nuclear proteins, leading to the conclusion that it was an insulin response sequence (IRS) (26, 27). Suwanichkul et al. (12, 28) identified two IRSs, IRSα and IRSβ, that mediate insulin inhibition of basal IGFBP-1 promoter activity. Goswami et al. (25) found that IRSβ overlaps the 3′ GRE, which was identified by footprinting and functional analysis of mutations in reporter gene assays. Subsequently, it was found that mutation of IRSα, which can bind forkhead/winged helix proteins, impairs induction by glucocorticoids, and it was suggested that these proteins may contribute to regulation of IGFBP-1 and PEPCK promoter activity by glucocorticoids and insulin (18, 29). IRSα and IRSβ are highly related to the PEPCK IRSs, and the IGFBP-1 IRSs also confer insulin inhibi-
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Insulin in H4IIE cells, which regulate PEPCK and IGFBP-1 as glucocorticoid-induced transcription and their inhibition by the IRSs of the PEPCK and IGFBP-1 promoters to basal as well as glucocorticoid-induced transcription. However, the effect of mutation of the IRSs is on regulation by insulin in the context of the endogenous PEPCK promoter and the roles of forkhead proteins and the IRS in mediating insulin inhibition of glucocorticoid-induced IGFBP-1 transcription have not been reported.

FKHR, another member of the winged helix family of transcription factors (34, 35), was shown to bind the IRSs present in the PEPCK and IGFBP-1 promoters as well as in other promoters (18, 29, 32, 36, 37), and mutations in the IRSs of the PEPCK or IGFBP-1 genes that reduce HNF3 binding also reduce glucocorticoid-induced transcription (18, 21, 32). However, transactivation by HNF3 proteins does not appear to be regulated by insulin (38). FKHR, another member of the winged helix family of transcription factors (34), binds specifically to IRSs (39), and IRS-dependent transactivation by FKHR can be suppressed by insulin (38, 40).

Previous studies showed that insulin activation of PKB and phosphorylation of FKHR are required for inhibition of basal IGFBP-1 transcription (38, 41, 42). However, the effect of mutation of the IRSs is on transcription from PEPCK and IGFBP-1 promoters. Our results show that gene- and activation-specific mechanisms of insulin inhibition exist to regulate PEPCK and IGFBP-1 transcription.

Figure 1. Effects of mutation of the IRSs and expression of FKHR on insulin inhibition of basal IGFBP-1-Luc activity. H4IIE cells were cotransfected with IGFBP-1-Luc reporters containing wild type (A) or mutated (B) IRSs within the IGFBP-1 promoter in the absence and presence of expression vectors for wild type FKHR or phosphorylation-defective FKHR (T/S/S), as described under “Materials and Methods.” Each precipitate was split into two dishes, and half of them were treated with 10 nM insulin (Ins) for the final 24 h of the experiment. Con, control. The results shown represent the mean ± S.E.M. of at least three independent experiments.

Figure 2. Effects of mutation of the IRSs and expression of FKHR on basal PEPCK-Luc activity. H4IIE cells were cotransfected with PEPCK-Luc reporters containing a wild type (A) or mutated (B) IRS within the PEPCK promoter in the absence and presence of expression vectors for wild type FKHR or phosphorylation-defective FKHR (T/S/S) as described under “Materials and Methods.” Each precipitate was split into two dishes, and half of them were treated with 10 nM insulin (Ins) for the final 24 h of the experiment. Con, control. The results shown represent the mean ± S.E.M. of at least three independent experiments.
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**Table I**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FKHR</th>
<th>T/S/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP-1-Luc</td>
<td>1.00</td>
<td>8.69</td>
<td>0.76</td>
</tr>
<tr>
<td>+Ins</td>
<td>0.10</td>
<td>0.72</td>
<td>0.11</td>
</tr>
<tr>
<td>+Dex</td>
<td>13.30</td>
<td>79.34</td>
<td>14.12</td>
</tr>
<tr>
<td>+Dex/Ins</td>
<td>0.22</td>
<td>2.51</td>
<td>0.59</td>
</tr>
<tr>
<td>mIRS-IGFBP-1-Luc</td>
<td>0.73</td>
<td>1.96</td>
<td>0.27</td>
</tr>
<tr>
<td>+Ins</td>
<td>0.28</td>
<td>0.43</td>
<td>0.03</td>
</tr>
<tr>
<td>+Dex</td>
<td>3.30</td>
<td>9.95</td>
<td>2.33</td>
</tr>
<tr>
<td>+Dex/Ins</td>
<td>0.40</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>PECK-Luc</td>
<td>1.00</td>
<td>4.28</td>
<td>0.52</td>
</tr>
<tr>
<td>+Ins</td>
<td>0.22</td>
<td>0.34</td>
<td>0.12</td>
</tr>
<tr>
<td>+Dex</td>
<td>20.83</td>
<td>50.33</td>
<td>11.38</td>
</tr>
<tr>
<td>+Dex/Ins</td>
<td>0.37</td>
<td>0.99</td>
<td>0.42</td>
</tr>
<tr>
<td>mIRS-PECK-Luc</td>
<td>1.03</td>
<td>2.66</td>
<td>0.70</td>
</tr>
<tr>
<td>+Ins</td>
<td>0.34</td>
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<tr>
<td>+Dex</td>
<td>6.98</td>
<td>21.74</td>
<td>6.23</td>
</tr>
<tr>
<td>+Dex/Ins</td>
<td>0.41</td>
<td>0.39</td>
<td>0.38</td>
</tr>
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</table>

(FB1-Luc) and mIRS-IGFBP-1-Luc (IRS,mut, in which both IRSA and IRSB are mutated) plasmids were previously described (41) as the wild type FKHR and PKB phosphorylation-defective FKHR (T/S/S) (38). Briefly, the IGFBP-1 promoter containing the SauI/Hgal fragment of the rat IGFBP-1 promoter, which extends 320 base pairs 5’ from the RNA cap site, was cloned immediately upstream of the luciferase cDNA in pGL2 (Promega) to produce IGFBP-1-Luc. The mIRS-IGFBP-1-Luc reporter was created using unique Nhel and BamHI restriction sites to replace IRSA and IRSB with mutated sequences (CAAAAACACT-TATTTTG to CACCGAGCAAGGCGTCA) as previously reported (41).

The wild type FKHR and T/S/S expression vectors contain the complete FKHR cDNA cloned into pAlterMax (41), and mutagenesis of the putative PKB phosphorylation sites at Thr-24, Ser-256, and Ser-319 to Gly and His-215 to Pro, was previously described (43). Mutation of FKHR sites mutated in T/S/S.

RESULTS

**Effects of FKHR on Basal IGFBP-1 and PECPK Promoter Activity**—To determine whether FKHR regulates basal expression of the IGFBP-1 and/or PECPK promoters through their IRS sequences, H4IIE cells were cotransfected with luciferase reporter vectors under the control of promoters with wild type and mutated IRSs and expression vectors for FKHR or FKHR-T/S/S (T/S/S). Expression of FKHR stimulated IGFBP-1-Luc activity, which was effectively inhibited by insulin (Fig. 1A). In contrast, expression of T/S/S, which cannot be phosphorylated by PKB, also stimulated IGFBP-1-Luc, but was only partially inhibited by insulin. Mutation of IRSA and IRSB in the IGFBP-1 promoter caused a decrease in basal luciferase activity (Fig. 1B) and also decreased insulin inhibition of basal activity by ~50%. Previous reports show that mutation of the IGFBP-1 IRSs abolishes insulin- or PKB-mediated regulation of basal IGFBP-1 activity in HepG2 cells (38, 41). Expression of either FKHR or T/S/S increased activity of mIRS-IGFBP-1-Luc, although to a lesser extent than for IGFBP-1-Luc, indicating that FKHR interacts with other sites in the IGFBP-1 construct when overexpressed in H4IIE cells. Insulin inhibited mIRS-IGFBP-1-Luc promoter activity stimulated by FKHR by 50% but not that stimulated by T/S/S. This difference in the effectiveness of insulin in suppressing T/S/S-enhanced transcription of the wild type and mIRS-IGFBP-1 promoters may indicate that another IRS-associated factor contributes to induction but not inhibition by insulin in H4IIE cells, or that insulin suppresses transactivation by IRS-associated FKHR in H4IIE cells through a mechanism that does not require phosphorylation of the FKHR sites mutated in T/S/S.

**PECPK-Luc activity** was inhibited by insulin and stimulated by expression of FKHR or T/S/S (Fig. 2A). As with IGFBP-1, expression of FKHR had no effect on insulin inhibition. However, in contrast to the pattern seen with IGFBP-1, expression of T/S/S had little effect on insulin inhibition, suggesting that phosphorylation of putative PKB sites in FKHR is not required for insulin inhibition of basal PECPK promoter activity. Also, mutation of the PECPK IRS had no effect on basal activity or its inhibition by insulin (Fig. 2B), indicating that the IRS is not required for inhibition of basal transcription.

**Fig. 3. Effects of IGFBP-1 IRS mutations and expression of FKHR on glucocorticoid-induced and insulin-inhibited IGFBP-1-Luc activity.** H4IIE cells were cotransfected with IGFBP-1-Luc reporter vectors containing wild type (A) or mutated (B) IRSs within the IGFBP-1 promoter in the absence and presence of expression vectors for wild type FKHR or phosphorylation-defective FKHR (T/S/S) as described under “Materials and Methods.” Each precipitate was split into four dishes, and half was treated with 0.5 μM dexamethasone (Dex) and/or 10 nM insulin (Ins) for the final 24 h of the experiment, as indicated. Con, control. The results shown represent the mean ± S.E.M. of at least three independent experiments.

**Fig. 4. Effects of mutation of the PECPK IRS and expression of FKHR on glucocorticoid-induced and insulin-inhibited PECPK-Luc activity.** H4IIE cells were cotransfected with PECPK-Luc reporter vectors containing wild type (A) or mutated (B) IRSs within the PECPK promoter in the absence and presence of expression vectors for wild type FKHR or phosphorylation-defective FKHR (T/S/S) as described under “Materials and Methods.” Each precipitate was split into four dishes, half of which were treated with 0.5 μM dexamethasone (Dex) and/or 10 nM insulin (Ins) for the final 24 h of the experiment, as indicated. Con, control. The results shown represent the mean ± S.E.M. of at least three independent experiments.
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stimulatory activity of FKHR or T/S/S was reduced in mIRS-PEPCK-Luc, but the ability of insulin to inhibit was unaffected. Together, these results indicate that the activity of the IGFBP-1 and PEPCk IRSs and their association with FKHR are context-dependent. The IGFBP-1 IRSs are crucial for strong FKHR activation, and the putative PKB phosphorylation sites in FKHR contribute to insulin inhibition of basal IGFBP-1 transcription. In contrast, FKHR stimulates PEPCk to a lesser extent than it does IGFBP-1, and neither the IRS nor phosphorylation of putative PKB sites are required for insulin inhibition of basal PEPCk promoter activity.

Insulin Inhibition of Glucocorticoid-induced IGFBP-1 and PEPCk Expression Occurs through an IRS-independent Mechanism(s)—Both the PEPCk and IGFBP-1 IRSs are positioned within glucocorticoid response units, and they have been suggested to disrupt the inducer complex to mediate insulin inhibition of glucocorticoid induction (18, 32, 33). To test this hypothesis, we examined the effect of expression of FKHR or T/S/S on glucocorticoid-induced activity of wild type and mIRS PEPCk and IGFBP-1 promoters (Table I, Figs. 3 and 4). Expression of FKHR or T/S/S enhanced both basal and dexamethasone-induced IGFBP-1 (Fig. 3A) and PEPCk (Fig. 4A) transcription. For either IGFBP-1 or PEPCk, mutation of the IRSs decreased dexamethasone induction by 33–67%, consistent with their proposed roles as accessory factors (17, 21, 24) regardless of whether cells were cotransfected with FKHR or not (Table I). Insulin inhibition of dexamethasone-induced transcription of either promoter was unaffected by mutation of the IRSs or of putative PKB phosphorylation sites in T/S/S. Insulin still inhibited the induction of mIRS-IGFBP-1 by dexamethasone, an effect also seen in HepG2 cells.2 Expression of FKHR or T/S/S reduced induction of IGFBP-1 by dexamethasone nearly as effectively as mutation of the IRS, but neither FKHR nor T/S/S had any effect upon induction of mIRS-IGFBP-1 by dexamethasone, an effect also seen in HepG2 cells.2 Expression of FKHR or T/S/S also reduced the extent of induction of PEPCk-Luc relative to control (Fig. 4B). These results indicate that endogenous factors may be displaced by overexpressed FKHR, as suggested by the recent studies of Hall et al. (51) and Ghosh et al. (52). Alternatively, attenuated induction of the mIRS promoter by FKHR may result from squelching through interactions with co-activators, as reported (53, 54). Together, these data indicate that an IRS-independent mechanism is utilized by insulin to inhibit glucocorticoid-induced IGFBP-1 or PEPCk transcription and that phosphorylation of putative PKB sites in FKHR is not required for this effect.

The Effect of FKHR on Basal IGFBP-1 and PEPCk Transcription Requires DNA Binding—Because the effect of FKHR on transcription was reduced but not eliminated by mutation of the IRS, we tested the activity of a modified form of FKHR in which critical residues within helix 3 of the DNA binding domain are mutated, and DNA binding is disrupted (H3-mut) (43). As shown in Fig. 5, H3-mut only slightly stimulated PEPCk-Luc activity and had no effect upon IGFBP-1-Luc activity, indicating that DNA binding is required for stimulation by FKHR. When the carboxyl-terminal activation domain of FKHR (amino acids 593–655) was fused to the Gal4 DNA binding domain and induction was tested using a luciferase reporter under the control of 5 Gal4 binding sites (5XGT-Luc), expression of the Gal4-FKHR fusion protein showed strong induction but no insulin inhibition, suggesting that the presence of the FKHR activation domain is all that is necessary for induction and that other domains within FKHR are required for inhibition of transactivation by insulin.

DISCUSSION

The present study shows that FKHR can interact with IRSs in the IGFBP-1 gene and that insulin can inhibit basal expression by antagonizing this interaction, whereas the PEPCk IRS can bind FKHR, but this is not essential for insulin inhibition of PEPCk promoter activity. Although the PEPCk and IGFBP-1 IRSs act as accessory factor sites within the GRUs, they are not required for insulin inhibition of glucocorticoid-induced transcription. Insulin inhibition of basal PEPCk expression and glucocorticoid-induced expression of either gene in H4IIE cells is not dependent upon IRSs or phosphorylation of putative PKB sites in FKHR. Thus, despite the fact that the PEPCk and IGFBP-1 IRSs act as accessory factor sites within the GRUs, they are not required for insulin inhibition of glucocorticoid-induced transcription. These data suggest that gene- and activation-specific mechanisms are employed by insulin to inhibit basal and hormone-induced transcription of these genes.

The IRS was first identified by testing candidate sequences for their ability to confer insulin inhibition upon a thymidine kinase promoter when inserted in the −50 position (26). An IRS, mutation of which abolished both insulin regulation and specific protein binding, was identified within AF2 of the GRU (26). In hindsight, it seems that this strategy led to identification of a binding site for FKHR and perhaps other closely related Fox(o) forkhead proteins (61), which, when evaluated in isolation, provide enhancement of basal transcription that can be inhibited by insulin. Subsequently, a much greater appreci-
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...Glucocorticoid-induced transcription of the PEPCK and IGFBP-1 genes, different mechanisms are required only the minimal PEPCK promoter (50). Here we show that insulin employs distinct mechanisms to inhibit basal and GRU-mediated IGFBP-1 gene expression. Thus, despite overall similarity in the promoter architecture and regulatory patterns of the PEPCK and IGFBP-1 genes, different mechanisms are used by insulin to inhibit basal and inducible expression of these genes. The utilization of distinct regulatory pathways to mediate regulation of gene transcription in a context- and mechanism-specific manner may be a more general phenomenon, indicating that a variety of transcription factors are regulated, perhaps by distinct insulin-signaling pathways.

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Addendum—While this manuscript was under review, Lechner et al. (Lechner, P. S., Croniger, C. M., Hakimi, P., Millward, C., Fekter, C., Yun, J. S., and Hanson, R. W. (2001) J. Biol. Chem. 276, 22675–22679) reported studies in transgenic mice showing that deletion of the PEPCK promoter sequences containing the IRS (AF2) limits glucocorticoid induction of PEPCK and IGFBP-1 genes, different mechanisms are used by insulin to inhibit basal and inducible expression of the two genes. The utilization of distinct regulatory pathways to mediate regulation of gene transcription in a context- and mechanism-specific manner may be a more general phenomenon, indicating that a variety of transcription factors are regulated, perhaps by distinct insulin-signaling pathways.

REFERENCES


3 S. Cichy, S. Guo, and T. Unterman, unpublished data.