A Nucleoprotein Complex containing CCAAT/Enhancer-binding Protein β Interacts with an Insulin Response Sequence in the Insulin-like Growth Factor-binding Protein-1 Gene and Contributes to Insulin-regulated Gene Expression*

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Highly related insulin response sequences (IRSs) mediate effects of insulin on the expression of multiple genes in the liver, including insulin-like growth factor binding protein-1 (IGFBP-1) and phosphoenolpyruvate carboxykinase (PEPCK). Gel shift studies reveal that oligonucleotide probes containing an IRS from the IGFBP-1 or PEPCK gene form a similar complex with hepatic nuclear proteins. Unlabeled competitors containing the IGFBP-1 or PEPCK IRS or a binding site for C/EBP proteins inhibit the formation of this complex. Antibody against C/EBPβ (but not other C/EBP proteins) super-shifts this complex, and Western blotting of affinity purified proteins confirms that C/EBPβ is present in this complex. Studies with affinity purified and recombinant protein indicate that C/EBPβ does not interact directly with the IRS, but that other factors are required. Gel shift assays and reporter gene studies with constructs containing point mutations within the IRS reveal that the ability to interact with factors required for the formation of this complex correlates well with the ability of insulin to regulate promoter activity via this IRS (r = 0.849, p < 0.01). Replacing the IRS in reporter gene constructs with a C/EBP-binding site (but not an HNF-3/forkhead site or cAMP response element) maintains the effect of insulin on promoter activity. Together, these findings indicate that a nucleoprotein complex containing C/EBPβ interacts with IRSs from the IGFBP-1 and PEPCK genes in a sequence-specific fashion and may contribute to the ability of insulin to regulate gene expression.

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Highly related insulin response sequences (IRSs) have been found to mediate effects of insulin on the expression of a number of genes in the liver, including phosphoenolpyruvate carboxykinase, insulin-like growth factor binding protein-1 (IGFBP-1), glucose-6-phosphatase, and apolipoprotein CIII (1–4). This observation has suggested that insulin may exert effects on the expression of multiple hepatic genes through a common mechanism (5). Signaling via phosphatidylinositol 3'-kinase is critical for the ability of insulin to suppress activity of the IGFBP-1, PEPCK, and Glu-6-Pase promoters (6–8) and we have reported that protein kinase B can mediate effects of insulin on basal IGFBP-1 promoter activity via an IRS downstream from phosphatidylinositol 3'-kinase (6). HNF-3/forkhead proteins can bind to oligonucleotide probes containing the insulin response sequences from the IGFBP-1 and PEPCK genes (9, 10). However, these interactions also involve sequences flanking the IRS, indicating that HNF-3 proteins are not likely to mediate sequence-specific effects of insulin on promoter activity via an IRS (10, 11). Recent reports have shown that another subgroup of forkhead/winged-helix transcription factors, including FKHR, FKHRL1, and AFX, can stimulate transcription through an IRS when they are overexpressed in cells, and that phosphorylation by protein kinase B can disrupt IRS-dependent transactivation by these proteins (12–16). However, studies to date have not demonstrated interaction between endogenous FKHR, FKHRL1, or AFX in nuclear extracts and an IRS in binding assays. Also, Hall et al. (17) have suggested that these forkhead family members may not interact with IRSs with the appropriate sequence-specificity to account for the ability of insulin to regulate promoter activity through an IRS in cells, indicating that other (as yet unidentified) factors also may contribute to the ability of insulin to suppress promoter function through an IRS (17).

CCAAT/enhancer-binding proteins (C/EBP) are enriched in the liver where they play an important role in the regulation of gene expression (18) and studies in knockout mouse models reveal that C/EBP proteins play a critical role in the regulation of hepatic glucose production during development and in fasting and insulin-deficient mice (19–22). Cell culture studies have shown that C/EBP proteins also are critically involved in the differentiation of adipocytes (23–25), indicating that they...
play an important role in the integrated regulation of metabolic processes (26). We have previously reported that a nucleoprotein complex that interacts with an IRS in the IGFBP-1 promoter may involve members of the C/EBP family of transcription factors, based on gel shift studies with an unlabeled competitor containing a known C/EBP-binding site (11). We now report that a similar complex also interacts with the IRS in the PEPCK gene and that this complex contains C/EBPβ. Gel shift and reporter gene studies with constructs containing point mutations within the IRS indicate that this complex interacts with an IRS in a sequence-specific fashion that correlates well with the ability of insulin to regulate promoter activity. Also, replacing the IRS with a consensus C/EBP-binding site (but not an HNF-3-binding site or CAMP response element (CRE)) maintains the effect of insulin on promoter activity. Taken together, these studies indicate that a nucleoprotein complex containing C/EBPβ can interact with IRSs in both the IGFBP-1 and PEPCK genes and may contribute to the ability of insulin to suppress promoter function. 

EXPERIMENTAL PROCEDURES

Materials and Expression Vectors—Antibodies against C/EBPβ (sc-61), C/EBPβ (sc-150), C/EBFβ (sc-151), ATF-1 (sc-270) which also recognizes CREB 1 p43 and CREM-1), ATF-3 (sc-188), ATF-4/C/EBPβ (sc-200), c-Jun (sc-44, which also recognizes JunB and JunD p39), c-Fos (sc-253, which also recognizes FosB, Fra-1, and Fra-2), Rb p110 (sc-50-G), NFκB p50 (sc-7178, which also recognizes NFκB p105), NFκB p60 (sc-7151), YY1 (sc-281), FKHR (sc-9809), FKHR1 (sc-9813), and the glucocorticoid receptor (sc-1003) were obtained from Santa Cruz Biotechnology. Antibody against HNF-3α and HNF-3β were provided by Dr. Robert Costa, and antiserum against HNF-3β was provided by Dr. K. Calame, and rabbit antiserum against the C-terminal peptide, 1

Partial Purification and Western Blotting of Nuclear Proteins—

Initial studies revealed that a 32P-labeled double-stranded oligonucleotide probe containing the IGFBP-1 response element (BP1) forms multiple nucleoprotein complexes with nuclear extracts prepared from rat H4IIE hepatoma cells (Fig. 1A, left panel). The formation of one complex (solid arrowhead) is inhibited by a 250-fold molar excess of an unlabeled oligonucleotide competitor containing a high affinity binding site for HNF-3β (Fig. 1A, right panel). Other oligos, not shown here, have shown that this complex contains HNF-3β, based on supershift studies (9). An unlabeled oligo containing the IRS and flanking sequences from the PEPCK gene partially inhibits the formation of this complex, consistent with studies indicating that this region of the PEPCK gene weakly interacts with HNF-3β/forkhead proteins (8, 9).

In contrast, the formation of a complex with lower mobility (Fig. 1, solid arrow) is inhibited effectively by both the BP1 and PEPCK oligos, but not by oligos containing binding sites for HNF-3/forkhead proteins, DBP, Sp1, or Oct1. A complex with similar mobility is formed when the oligo containing the IRS

RESULTS

Initial studies revealed that a 32P-labeled double-stranded oligonucleotide probe containing the IGFBP-1 insulin response element (BP1) forms multiple nucleoprotein complexes with nuclear extracts prepared from rat H4IIE hepatoma cells (Fig. 1A, left panel). The formation of one complex (solid arrowhead) is inhibited by a 250-fold molar excess of an unlabeled oligonucleotide competitor containing a high affinity binding site for HNF-3β (Fig. 1A, right panel). Other oligos, not shown here, have shown that this complex contains HNF-3β, based on supershift studies (9). An unlabeled oligo containing the IRS and flanking sequences from the PEPCK gene partially inhibits the formation of this complex, consistent with studies indicating that this region of the PEPCK gene weakly interacts with HNF-3β/forkhead proteins (8, 9).

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and flanking sequences from the PEPCK promoter is used as a probe (Fig. 1A, right panel, open arrow), and the formation of this complex also is inhibited by competitors containing the IGFBP-1 insulin response element or PEPCK IRS, but not by other competitors. This indicates that this competition is specific for C/EBP proteins in complexes formed with BP1 and PEPCK probes. A, competitive binding studies. Nuclear proteins were prepared from H4IIE hepatoma cells (10 μg/lane) or rat liver (2.5 μg) in the presence of 50 μg/ml poly(dI-dC) prior to electrophoresis and autoradiography, as before. (Note: lanes 1–4 of the left panel were included in a previous publication (9) and are presented with permission of Academic Press.) B, supershift studies with antibodies for C/EBP proteins. Nuclear proteins from rat liver (1 μg/lane) were preincubated with/without the competitor containing a high affinity HNF-3-binding site and then with polyclonal antibodies specific for C/EBPβ, C/EBPδ, or C/EBPβ prior to incubation with the labeled BP1 (left) and PEPCK (right) probes in the presence of 5 μg/ml poly(dI-dC).

petitors, suggesting that C/EBP proteins may contribute to the formation of this complex. An excess of the C/EBP oligo (but not other competitors) also inhibits the formation of the corresponding complex that is formed with the probe containing the PEPCK IRS (not shown). As shown in Fig. 2B, polyclonal goat antibody against C/EBPβ (but not C/EBPα or C/EBPβ) supershifts the complex that is formed by liver nuclear proteins and either the BP1 (left panel) or PEPCK (right panel) probe, indicating that C/EBPβ is present in this complex.

The sequences flanking IRSs in the BP1 and PEPCK probes are not conserved (Fig. 1), suggesting that the formation of this complex involves direct interaction with IRSs in these probes. To explore this possibility, we created an oligonucleotide probe containing a single IRS (CAAAACA) which has been shifted upstream from its location in the BP1 probe to disrupt potential interactions with flanking sequences (ΔIRS.1), or where the IRS is returned to its initial location, but flanking sequences are modified (ΔIRS.2). As shown in Fig. 3A, the ΔIRS.1 and ΔIRS.2 probes form a complex with similar mobility. The formation of this complex is inhibited by an excess of unlabeled oligos containing an IRS (ΔIRS.1 and ΔIRS.2), but not by oligos where the IRS is mutated (ΔIRS.1M and ΔIRS.2M) (Fig. 3A), supporting the concept that interaction with the IRS is required for the formation of this complex. Supershift studies performed in the presence of an excess of the ΔIRS.1M competitor reveal that the
FIG. 3. Interaction of the C/EBPβ-containing complex with an IRS. A, gel shift with ΔIRS.1 probe. Nuclear proteins prepared from rat liver (1 μg/lane) were preincubated with/without a 100-fold excess of unlabeled competitors and 5 μg/ml poly(dI-dC)poly(dI-dC) prior to the addition of labeled ΔIRS.1 or BP1 probes. The ΔIRS.1 oligo contains a single IRS (CAAAAA), which has been shifted 3 bp 5’ from its location in the BP1 probe to disrupt potential interactions with flanking sequences. The IRS is replaced by an unrelated sequence in the ΔIRS.1M oligo. B, supershift with the ΔIRS.1 probe. Nuclear proteins from rat H4IE (10 μg/lane) (left panel) or human HepG2 hepatoma cells (10 μg/lane) (middle panel) or rat liver (1 μg) (right panel) were preincubated with a 100-fold excess of the unlabeled ΔIRS.1M oligo to inhibit the formation of complexes which do not involve interaction with the IRS, and then with antibodies against C/EBPβ, C/EBPδ, or C/EBPα, prior to incubation with the labeled ΔIRS.1 probe.

ΔIRS.1 probe forms a complex containing C/EBPβ with nuclear proteins prepared from H4IE or HepG2 hepatoma cells (10 μg) or rat liver (1 μg) (Fig. 3B). Studies with the ΔIRS.2 probe yielded similar results (not shown). These results support the concept that the formation of this complex containing C/EBPβ involves interaction with the IRS.

To characterize this complex further, we partially purified liver nuclear proteins forming this complex by chromatography with heparin-Sepharose and three rounds of DNA-affinity chromatography using a matrix constructed with oligonucleotides containing an array of 3 IRSs and monitored binding activity in column eluates by gel shift assay (Fig. 4A). Goat polyclonal antibody against the C-terminal region of C/EBPβ (2 μg/lane) supershifts the major complex formed with partially purified proteins and the ΔIRS.1 probe (Fig. 4B, left panel), similar to results with crude nuclear extracts. Studies with 2 μg/lane rabbit polyclonal antiserum against the C-terminal region of C/EBPβ (C/EBPβC,CT) also supershifts this complex (Fig. 4B, right panel), although not as completely as 2 μg of the purified goat antibody. Interestingly, antisera against the N-terminal region of C/EBPβ (C/EBPβN) does not disrupt or supershift this complex, suggesting that this region of C/EBPβ may be masked. Western blotting indicates that 35-, 32-, and 14-kDa forms of C/EBPβ (30) are present in these partially purified proteins (Fig. 4C).

As shown in Fig. 5A, competitive binding studies revealed that a 10-fold molar excess of the unlabeled ΔIRS.1 oligo is sufficient to effectively inhibit the ability of partially purified proteins to form this complex with the ΔIRS.1 probe (solid arrow). Higher titers of a competitor containing a consensus C/EBP-binding site (TTGCGCAA; ΔC/EBP) are required to inhibit the ability of the ΔIRS.1 probe to form this complex. This result indicates that some factor(s) required for the formation of this complex interacts preferentially with the IRS compared with a consensus C/EBP-binding site.

Also shown in Fig. 5A, partially purified proteins form a complex with higher mobility when the oligo containing a consensus C/EBP-binding site is used as a probe (open arrow), and the formation of this complex is competitively inhibited by a 10-fold excess of
D has been replaced by a consensus C/EBP-binding site (TTGCGCAA; autoradiography. D recombinant GST-CHOP or GST, prior to incubation with the labeled probe containing a single IRS (Fig. 5A), a recombinant GST fusion protein containing CHOP, a member of the C/EBP family, was preincubated with/without unlabeled competitors prior to incubation with a 32P-labeled probe containing the IGFBP-1 insulin response element (Fig. 6). Gel shift studies performed under conditions previously found to optimize the binding of C/EBP proteins to the PEPCK probe (10) reveal that recombinant full-length (C/EBP) or truncated (C/EBP) C/EBP probe was incubated with recombinant full-length (C/EBPβ1–276; left panel) or truncated (C/EBPβ132–276; right panel) C/EBPβ in the presence of poly(d-l-c)-poly(d-l-c) under our standard conditions. Bound and free probe were resolved by native gel electrophoresis at 4 °C, as in Figs. 1–5.

Several transcription factors have been reported to interact with C/EBPβ and form nucleoprotein complexes, including ATF4 (also known as CREB-2) (33), c-Jun and c-Fos (34), YY1 (35), NFκBβ (36, 37), retinoblastoma (Rb) protein p110 (38), and the glucocorticoid receptor (39). As shown in Fig. 7A, antibodies which recognize ATF1 (and CREB-1 and CREM-1), ATF3, ATF4/CREB-2, c-Jun (and JunB and JunD), c-Fos (and FosB, Fra-1, and Fra-2), Rb p110, YY1, NFκBβ p50 (and p105) or p65 do not disrupt or supershift this complex. Antibodies against the human glucocorticoid receptor, HNF-3α, -3β, and -3γ, or against the N-terminal region of FKHR and FKHRL1, which are expressed in the liver, also do not supershift or disrupt the formation of this complex or other complexes formed with this probe (data not shown).

Previous studies indicate that a complex containing a ~20-kDa protein and C/EBPα interacts with probes containing either the CRE or the IRS in the PEPCK promoter, and that this complex has ~30-fold greater affinity for the CRE compared with the PEPCK IRS (29). As shown in Fig. 7B, unlabeled oligos containing the PEPCK CRE (ΔCRE) or the CRE together with flanking sequences from the PEPCK gene (CRE) do not inhibit the formation of the complex containing C/EBPβ that is formed with partially purified proteins and the ΔIRS.1 probe. This indicates that formation of this complex does not require a factor that interacts preferentially with the PEPCK CRE.

To determine whether the complex containing C/EBPβ might

**FIG. 5. Interaction of partially purified proteins with oligos containing an IRS or consensus C/EBP-binding site.** A, competitive binding studies. Partially purified proteins were preincubated with/without unlabeled competitors prior to incubation with a 32P-labeled probe containing a single IRS (ΔIRS.1) or a probe where the IRS has been replaced by a consensus C/EBP-binding site (TTGCGCAA; Δ/EBP). B, effect of recombinant GST-CHOP. Partially purified proteins (2 μl) were preincubated with 500 ng of bacterially expressed recombinant GST-CHOP or GST, prior to incubation with the labeled Δ/EBP or ΔIRS.1 probe, then loaded for native gel electrophoresis and autoradiography.

**FIG. 6. Gel shift studies with recombinant C/EBPβ.** A, gel shift with poly(d-g-d)-poly(d-g-d). Initial gel shift studies with recombinant C/EBPβ were performed under conditions which optimize interactions between C/EBP proteins and probes containing the PEPCK IRS and flanking sequences (10, 29). Recombinant full-length C/EBPβ (20 ng) was preincubated with/without a 250-fold excess of an unlabeled oligonucleotide competitor containing a consensus C/EBP-binding site, and then with probes containing the C/EBP probe (C/EBP), the IGFBP-1 insulin response element (BP1), or the PEPCK IRS in the presence of poly(d-g-d)-poly(d-g-d) at 4 °C, then loaded for native gel electrophoresis at 22 °C. B, standard gel shift conditions. The labeled ΔIRS.1 or Δ/EBP probe was incubated with recombinant full-length (C/EBPβ1–276) or truncated (C/EBPβ132–276) C/EBPβ in the presence of poly(d-l-c)-poly(d-l-c) under our standard conditions. Bound and free probe were resolved by native gel electrophoresis at 4 °C, as in Figs. 1–5.

**FIG. 7. Antibodies recognizing...** Several transcription factors have been reported to interact with C/EBPβ and form nucleoprotein complexes, including ATF4 (also known as CREB-2) (33), c-Jun and c-Fos (34), YY1 (35), NFκBβ (36, 37), retinoblastoma (Rb) protein p110 (38), and the glucocorticoid receptor (39). As shown in Fig. 7A, antibodies which recognize ATF1 (and CREB-1 and CREM-1), ATF3, ATF4/CREB-2, c-Jun (and JunB and JunD), c-Fos (and FosB, Fra-1, and Fra-2), Rb p110, YY1, NFκBβ p50 (and p105) or p65 do not disrupt or supershift this complex. Antibodies against the human glucocorticoid receptor, HNF-3α, -3β, and -3γ, or against the N-terminal region of FKHR and FKHRL1, which are expressed in the liver, also do not supershift or disrupt the formation of this complex or other complexes formed with this probe (data not shown).

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To determine whether the complex containing C/EBPβ might
proteins were preincubated with specific antibodies against C/EBP
A, partially purified proteins prior to incubation with the32P-labeled
D probe by 66%, based on PhosphorImaging. Replacing residue 6
mutations of individual residues within the IRS. A, dose response study. Partially purified proteins were preincubated with a 2–50-fold molar excess of unlabeled oligonucleotide competitors prior to incubation with the ΔIRS.1 probe. The sixth residue in the IRS (CAAAACA) is replaced in the ΔIRS.1m6 competitor (CAAAAGa). B, competitive binding studies. Partially purified proteins (2 μl/lane) were preincubated with a 5-fold molar excess of the unlabeled ΔIRS.1 oligo or oligos where individual residues within the IRS are altered one at a time (ΔIRS.1m1–ΔIRS.1m7) prior to the addition of the labeled ΔIRS.1. The sequence of the each altered IRS is shown in Table I.

Fig. 8. Mutation of individual residues within the IRS. A, dose response study. Partially purified proteins were preincubated with a 2–50-fold molar excess of unlabeled oligonucleotide competitors prior to incubation with the ΔIRS.1 probe. The sixth residue in the IRS (CAAAACA) is replaced in the ΔIRS.1m6 competitor (CAAAAGa). B, competitive binding studies. Partially purified proteins (2 μl/lane) were preincubated with a 5-fold molar excess of the unlabeled ΔIRS.1 oligo or oligos where individual residues within the IRS are altered one at a time (ΔIRS.1m1–ΔIRS.1m7) prior to the addition of the labeled ΔIRS.1. The sequence of the each altered IRS is shown in Table I.

molar excess of unlabeled oligos to interact with factors which are required for the formation of this complex. In contrast, altering residues 1, 2, or 7 or the IRS does not impair the ability of unlabeled oligos to inhibit the formation of this complex. As shown in Table I, altering residues 3, 4, 5, or 6 also reduces the ability of insulin to inhibit promoter activity via an IRS in reporter gene studies. As shown in Fig. 9, the ability of oligonucleotides containing point mutations within the IRS to interact with factors required for the formation of this complex correlates well with the ability of reporter gene constructs containing the same mutations to mediate inhibitory effects of insulin on promoter activity (r = 0.849, p < 0.01). Together, these results indicate that this complex interacts with the IRS in a sequence-specific fashion consistent with the concept that this complex may contribute to the ability of insulin to inhibit promoter activity via an IRS.

Since C/EBPβ is present in this complex, we next asked whether C/EBP proteins themselves might contribute to the ability of insulin to inhibit promoter activity. As shown in Table II, the ΔIRS.1 reporter gene construct contains a single IRS located ~100 bp upstream from the transcription initiation site, and insulin treatment reduces promoter activity in this construct by 55%. Substituting a single residue (ΔIRS.1m6) or several residues (ΔIRS.1M) within the IRS disrupts the ability of insulin to suppress promoter activity, indicating that this effect of insulin is mediated via the IRS, as previously reported (6). Placing a consensus C/EBP-binding site at the same location (ΔC/EBP) partially maintains the ability of insulin to inhibit promoter activity, similar to an IRS. Placing a consensus HNF-3 site (ΔHNF-3) or a CRE (ΔCRE) at this location does not maintain the ability of insulin to suppress promoter activity, demonstrating that this effect is specific. Control studies with FKHR expression and antisense vectors (12) confirm that FKHR forkhead proteins do not interact with this site and that phosphorylation of FKHR is not required for insulin to regulate promoter activity via this C/EBP-binding site (data not shown), indicating that the ability of insulin to regulate promoter activity via this C/EBP-binding site does not require interaction with FKHR-related forkhead proteins.

Placing an IRS (but not a mutated sequence) 3 bp further downstream (ΔIRS.2 versus ΔIRS.2M) or ~230 bp further upstream (ΔIRS.331 versus ΔIRS.331.m6) to disrupt potential interactions with flanking sequences also maintains the ability
Gel shift studies were performed using partially purified nuclear proteins and the ΔIRS.1 oligonucleotide probe containing a single IRS (CAAAAACA). The ability of a 5-fold molar excess of unlabeled oligonucleotides where a single residue within the IRS was altered was evaluated (Fig. 7B) and the amount of radioactive probe bound in the presence (B) and absence (B₀) of competitor was measured by phosphorimaging. To assess the effect of altering the sequence of the IRS on the ability of insulin to suppress promoter activity, we performed transient transfection assays in HepG2 cells using a luciferase reporter gene construct which contains 320 bp of the IGFBP-1 promoter. The insulin response element was removed and replaced by a single IRS (CAAAAACA) located −104 to −110 5’ to the transcription initiation site (ΔIRS.1), or a modified IRS where a single residue has been altered (ΔIRS.1M1 and ΔIRS.1M6), as previously reported (6). Cells were treated with serum-free medium with/without 100 nM insulin for 18 h prior to lysis and analysis of luciferase activity, as before (6, 11). (Note: the effect of insulin on promoter activity in HepG2 cells transfected with these reporter gene constructs was reported previously (11) and is presented in this table and in Fig. 9 with the permission of the American Society for Biochemistry and Molecular Biology.)

| Construct     | Binding activity | Insulin % inhibition |
|---------------|------------------|----------------------|
| ΔIRS.1        | ATGGAAAAACACTT   | 0.34                 |
| ΔIRS.1M1      | ATGGAAAAACACTT   | 0.37                 |
| ΔIRS.1M2      | ATGGAAAAACACTT   | 0.34                 |
| ΔIRS.1M3      | ATGGAAAAACACTT   | 0.52                 |
| ΔIRS.1M4      | ATGGAAAAACACTT   | 0.94                 |
| ΔIRS.1M5      | ATGGAAAAACACTT   | 1.00                 |
| ΔIRS.1M6      | ATGGAAAAACACTT   | 0.95                 |
| ΔIRS.1M7      | ATGGAAAAACACTT   | 0.12                 |

**Table II**

Effect of insulin on promoter activity mediated via a C/EBP site

HepG2 cells were transfected with luciferase reporter gene constructs containing a functional IRS (ΔIRS.1, ΔIRS.2, or ΔIRS.331) or altered sequences (ΔIRS.1M1, ΔIRS.2M, or ΔIRS.331M0) or constructs where the IRS has been replaced by a CRE (ΔCRE) or a consensus binding site for HNF-3/forkhead (ΔHNF-3) or C/EBP (ΔC/EBP, ΔC/EBP.2, or ΔC/EBP.331) proteins. Transfected cells were refed with serum-free medium with/without 100 nM insulin 18 h prior to lysis and analysis of luciferase activity. The effect of insulin on promoter activity is expressed as percent inhibition relative to serum-free control (mean ± S.E.).

| Construct        | Insulin % inhibition |
|------------------|----------------------|
| ΔIRS.1           | 53 ± 4               |
| ΔIRS.1M1         | 58 ± 5               |
| ΔC/EBP           | 50 ± 5               |
| ΔHNF-3           | 55 ± 5               |
| ΔCRE             | 35 ± 4               |
| ΔIRS.2           | 17 ± 4               |
| ΔIRS.2M          | 34 ± 5               |
| ΔIRS.331         | 33 ± 6               |
| ΔIRS.331M4       | 32 ± 6               |
| ΔC/EBP.2         | 26 ± 7               |
| ΔC/EBP.331       | 27 ± 6               |

**DISCUSSION**

To understand specific mechanisms that may mediate effects of insulin on gene expression via an IRS, we examined nucleoprotein complexes that interact with oligonucleotide probes containing IRSs from either the IGFBP-1 or PEPCK gene. Previous studies have shown that recombinant C/EBP proteins can bind directly to oligonucleotide probes containing the PEPCK IRS, but not the IGFBP-1 insulin response element (10), and we confirmed this result. Direct binding of C/EBP proteins to the PEPCK IRS is thought to require interaction with flanking sequences and mutation of these sequences does not disrupt the effect of insulin (10). These observations suggested that C/EBP proteins are not likely to be involved in mediating the effect of insulin on promoter activity via an IRS (10). In contrast, we find that C/EBPβ is present in a complex of proteins that interacts with residues within an IRS that are critical for the effect of insulin, and that sequences flanking the IRS are not essential for the formation of this complex. In addition, replacing the IRS with a consensus C/EBP-binding site at the same location also confers an effect of insulin on promoter activity (24, 40). Studies in knockout mouse models show that C/EBP does not disrupt the effect of insulin (10). These observations support the notion that insulin regulates gene expression via an IRS to mediate the effect of insulin on promoter activity via an IRS. C/EBP proteins play an important role in the regulation of gene expression in the liver (18) and other insulin-responsive tissues (24, 40). Studies in knockout mouse models show that both C/EBPα and C/EBPβ contribute to the regulation of hepatic glucose production (20, 21, 41). C/EBPα contributes to the regulation of gluconeogenesis, and C/EBPβ contributes to the regulation of the PEPCK promoter by thyroid hormone, glucocorticoids, and cAMP agonists (43, 44). A recent report by Yeagley et al. (45) indicates that C/EBP proteins also play an important role in mediating effects of insulin on cAMP stimulated activity.
the PEPCK promoter independent of the PEPCK IRS. Together, these findings indicate that C/EBP proteins play an important role in the multihormonal regulation of hepatic gene expression.

Competitive binding and functional studies with constructs containing point mutations within the IRS revealed that 4 residues (AAAC) are essential for interactions with factors required for the formation of this complex and for mediating effects of insulin on promoter activity. Recent studies by Hall et al. (17) indicate that several of these residues (AAC) also are critical for mediating effects via the IRS in the PEPCK promoter in cAMP/dexamethasone-stimulated H4IIE hepatoma cells, supporting the concept that similar factors may interact with the PEPCK IRS and contribute to the ability of insulin to regulate that gene. Of note, the consensus HNF-3-binding site (AAACAAACATT) we introduced into a reporter gene construct (ΔHNF-3) also contains this core sequence, but does not confer the ability of insulin to suppress promoter activity. We have reported that the ability of IRSs in the IGFBP-1 and PEPCK genes to bind HNF-3 proteins is relatively weak compared with a known high affinity HNF-3-binding site (9). Presumably, high affinity binding of HNF-3 proteins to this consensus sequence may prevent interaction with other factors that are required to mediate the effect of insulin. Based on the present study, we speculate that interactions between HNF-3 proteins must be relatively weak to permit IRSs to interact with other factors that are critical for the ability for insulin to regulate gene expression in liver-derived cells, and that variations in sequences flanking the AAAC core are important in determining the relative affinity with which IRSs interact with insulin-responsive and/or blocking factors.

In this context, it is important to note that this core sequence also is conserved in a recently identified consensus binding sequence for FKHR-related forkhead proteins (including FKHR, FKHL1, AFX, and DAF-16) (46), which are thought to contribute to effects of insulin on gene expression (12–16). Taken together with the results of the present study, these observations suggest the interesting possibility that multiple factors, including forkhead proteins and the complex containing C/EBPβ, may interact with this core AAAC sequence and contribute to the ability of insulin to regulate promoter activity via an IRS. This concept also is suggested by previous studies indicating that IRSs from the apolipoprotein CIII and tyrosine aminotransferase genes also can interact with factors required for the formation of this C/EBPβ-containing complex (11), but that another IRS from the IGFBP-1 promoter (IRSB) does not (11).

Previous studies have shown that C/EBPβ may interact with and form nucleoprotein complexes with a number of factors, including ATF-4 (CREB-2), c-Jun, and c-Fos, Rh p110, and NFκB proteins and the glucocorticoid receptor. However, antibodies against these factors fail to disrupt or supershift the C/EBPβ-containing complex which interacts with the IRS. FKHR-related forkhead transcription factors are expressed in the liver and can interact directly with IRSs in the IGFBP-1 and PEPCK genes (12, 14, 15, 47, 48) and it is interesting to speculate that C/EBPβ may interact with these forkhead proteins to form a complex with the IRS. Unlabeled competitors containing the high affinity HNF-3-binding site from the transthyretin gene have been reported to inhibit the binding of recombinant FKHR to the IGFBP-1 insulin response element (47). However, a competitor containing this HNF-3-binding site failed to inhibit the formation of this C/EBPβ containing complex (Figs. 1 and 2). Also, antibodies against HNF-3/forkhead proteins, FKHR and FKHL1 (which also are expressed in liver cells), also failed to detect forkhead proteins in this complex, suggesting that forkhead proteins are not required for the formation of this complex. However, this negative results does not entirely exclude this possibility, since epitopes required for recognition by these antibodies may be masked by the formation of this complex. At the same time, the observation that a consensus C/EBP-binding site mediates effects of insulin on promoter activity similar to an IRS indicates that C/EBP proteins may contribute directly to the ability of insulin to suppress promoter activity via a mechanism that is independent of forkhead proteins, and studies reported in an accompanying paper (48) support this concept.

At the same time, it is important to note that a C/EBP site is only partially as effective in mediating an effect of insulin on promoter activity compared with a recognized IRS (Table II). In related studies, we found that insulin suppresses transactivation by C/EBPβ, but not C/EBPα when these proteins are expressed in HepG2 cells (48). Since both C/EBPα and C/EBPβ are expressed in HepG2 cells (49, 50), C/EBPα may limit the ability of insulin to suppress promoter activity through a consensus C/EBP-binding site (which can bind hetero- and homodimers of C/EBP proteins containing C/EBPα and/or C/EBPβ) compared with an IRS (which interacts with a complex containing C/EBPβ, but not C/EBPα). Alternatively, other factors, including forkhead proteins, may be required to mediate the full effect of insulin on promoter activity via an IRS in HepG2 cells.

In this context, it is interesting to note that the ability of probes containing an IRS to form this complex is enhanced in nuclear extracts prepared from rat liver compared with extracts prepared from H4IIE and HepG2 hepatoma cells. Since levels of C/EBP proteins are reduced in hepatoma cells lines compared with normal liver (49),2 nucleoprotein complexes containing C/EBPβ may play a more prominent role in mediating effects of insulin on hepatic gene expression in vivo than might be apparent in studies performed in these transformed cells.

In summary, the results of the present study indicate that a nucleoprotein complex containing C/EBPβ can interact with IRSs in the IGFBP-1 and PEPCK genes. Based on competitive binding data and studies with recombinant proteins, it appears that C/EBPβ does not interact directly with the IRS in the IGFBP-1 promoter, but that some other factor(s) is required. The ability of the IRS to interact with factors required for the formation of this complex correlates well with the ability of an IRS to mediate inhibitory effects of insulin on promoter activity, and replacing the IRS with a consensus C/EBP-binding site maintains this effect of insulin. Additional studies are required to identify the other factor(s) required for the formation of this complex, and to examine the relative role that this nucleoprotein complex and C/EBP proteins play in mediating effects of insulin on hepatic gene expression.

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