Identification of an Insulin-responsive Element in the Rat Insulin-like Growth Factor-binding Protein-3 Gene*

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The hepatic expression and serum levels of insulin-like growth factor-binding protein-3 (IGFBP-3) are decreased in insulin-dependent and insulin-resistant diabetes. Insulin increases hepatic IGFBP-3 expression by enhancing gene transcription. This report identifies sequences within the IGFBP-3 promoter that are necessary and sufficient for the response to insulin in hepatic nonparenchymal cells. By transient transfection, we mapped the insulin response element to the −1150 to −1124 base pair (bp) region of the rat IGFBP-3 promoter. Three tandem repeats of the −1150 to −1117 bp region conferred insulin responses in a heterologous promoter. Gel shift analyses revealed a 3-fold increase in DNA-protein complex formation with nuclear extracts obtained from insulin-stimulated nonparenchymal cells compared with cells incubated without insulin and revealed 2–4-fold decrease in complex formation with nuclear extracts obtained from the livers of streptozotocin-diabetic rats compared with control rats. Mutational analysis of this 34-bp region showed a core sequence of 10 bp (−1148 to −1139) that is critical for interaction with insulin-induced trans-acting factors. Southernwestern blotting revealed a −90-kDa protein that was increased 2–3-fold by the addition of insulin. Thus, we have identified cis-acting DNA sequences that are responsible for regulation of IGFBP-3 transcription by insulin and essential for binding of insulin-responsive nuclear factors.

Insulin-like growth factors I and II (IGF-I and -II)1 are peptides that have insulin-like metabolic and trophic effects and mediate some of the peripheral actions of growth hormone (1). The actions of IGFs are modulated by a family of six IGF-binding proteins (IGFBPs), which have different tissue distribution and production sites (2, 3). Most of the circulating plasma IGF-1 and IGF-II is associated with IGFBP-3 and an acid-labile subunit, constituting a −150-kDa complex, which serves as a reservoir for IGFs (4). Formation of this large molecular weight complex limits the access of IGFs to tissues and prevents the hypoglycemic effects of IGFs (5). Recent studies also suggest that the IGFBPs actively modulate the mitogenic and metabolic actions of IGFs in the cellular microenvironment (6–9).

The mechanisms by which IGFBP-3 is regulated are complex. IGFBP-3 may undergo post-translational processing to yield various proteolytically cleaved, phosphorylated, and glycosylated products (8–14). These processes have been shown to alter the binding of IGFBP-3 to the acid-labile subunit and cell surfaces and affect the affinity of IGFBP-3 for IGFs (15). IGFBP-3 can also associate with the cell surface and extracellular matrix; dissociation of cell-associated IGFBP-3 is one mechanism by which IGF-I increases release of IGFBP-3 into conditioned medium by fibroblasts and breast cancer cells (16, 17). While the post-translational regulation of IGFBP-3 appears to be important, IGFBP-3 is also regulated at the level of gene transcription.

The IGFBPs are structurally homologous, with strict conservation of the 18 cysteine residues clustered at the NH2 and COOH termini of the proteins (18). Despite their structural similarities, however, the IGFBPs differ in their pattern of developmental and hormonal regulation. IGFBP-1 and IGFBP-3 have been studied most extensively. While the serum protein and liver mRNA levels of IGFBP-1 reach the highest levels during fetal life and the neonatal period, the protein levels of IGFBP-3 in serum and liver mRNA levels are highest during puberty and adult life (19). Furthermore, IGFBP-1 levels decrease in the presence of anabolic hormones such as insulin and growth hormone, while IGFBP-3 levels increase in the presence of these hormones (20, 21). The IGFBP-1 and the IGFBP-3 genes are contiguously arranged in a tail-to-tail fashion within chromosome 7, separated by only 20 kilobase pairs of DNA (22). The juxtaposition of the genes and the high expression of both genes within the liver could theoretically allow cellular factors to regulate the genes similarly in a given physiological condition. To explain the markedly different responses of these structurally related proteins, there is interest in determining whether different sets of transcription factors regulate the promoter activity of these genes. At present, the cis-elements required for basal expression and insulin-mediated activity of the IGFBP-1 gene have been reported (23–26), while the cis-elements required for development- and hormone-mediated activity of the IGFBP-3 promoter have yet to be identified.

Our previous investigations showed that IGFBP-3 mRNA is an abundant transcript in the liver and is secreted by the Kupffer and sinusoidal endothelial cells (27). Through sequential collagenase/Pronase treatment, we were able to isolate nonparenchymal cells that express IGFBP-3 and maintain the phagocytic function of Kupffer cells, and we have used this cultured cell model to study the mechanisms involved in the

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1 The abbreviations used are: IGF, insulin-like growth factor; IGFBP, IGF-binding protein; rIGFBP, rat IGFBP; IRE, insulin response element; bp, base pair(s); nt, nucleotide(s); PEPCK, phosphoenolpyruvate carboxykinase.
hormonal regulation of IGFBP-3. We showed previously that insulin increased IGFBP-3 expression by stimulating the rate of gene transcription rather than through stabilization of mRNA transcripts (28). In the present study, we define the cis-acting sequences that mediate the positive effects of insulin on IGFBP-3 transcription, and recognize insulin-responsive factors in nuclear extracts. By transient transfection, we mapped the insulin response element (IRE) in the rIGFBP-3 gene to the region spanning −1150 to −124 bp. In gel mobility shift analyses, the IRE exhibited increased DNA-protein complex formation with nuclear extracts obtained from cells exposed to insulin compared with cells not exposed to insulin and decreased complex formation with extracts from diabetic compared to normal rat livers. Southwestern blotting revealed association of the IGFBP-3 IRE region with 90- and 70-kDa nuclear factors; the 90-kDa factor appears to be hormone-responsive and could contribute to regulation of IGFBP-3 transcription by insulin.

EXPERIMENTAL PROCEDURES

Materials—The reagents listed were obtained from the following sources: collagenase type 1 from Worthington; Pronase-E from Chem; type I rat tail collagen, human transferrin, fetal bovine serum, and Williams’ E medium from Sigma; Lipofectin reagent, human recombinant insulin, medium 199, and deoxyribonuclease 1 (DNase I) from Life Technologies, Inc.; [γ-32P]ATP from Amersham Corp. All restriction enzymes were from New England Biolabs (Beverly, MA).

Liver Nonsympathetic Cell Cultures and DNA Transfection—Livers from 180–200-g male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were perfused sequentially with 0.1% Pronase-E and 0.05% collagenase in situ and then incubated with 0.08% Pronase-E in 100 ml of Dulbecco’s modified Eagle’s medium/F12, pH 7.4, at 37°C for 30 min. Cells were centrifuged, washed 3 times, and plated on 6-well collagen-coated culture plates at 1–2 × 10^6 cells/well. After overnight incubation, cultures were maintained with daily changes of medium, 20% fetal bovine serum containing medium 199 on day 2, and serum-free media thereafter.

Transient transfections of chimeric constructs containing various deletions of rat IGFBP-3 promoter regions attached to the luciferase reporter gene were undertaken in liver nonparenchymal cells on day 3 of culture. Lipofectin reagent and DNA complexes were mixed at a 15 μg to 2.5 μg ratio and incubated with the cells aerobically. Medium was replaced with serum-free medium 199 on day 4, with or without the addition of 10 μM insulin for 24 h, and cell extracts were assayed on day 5 for gene activity using the luciferase assay system (manufacturer’s recommended protocol from Promega) and measured by a Microsorb 100 luminesimeter (LKB Wallac, Turku, Finland). All readings were within the linear range of the instrument when compared with known luciferase concentrations.

DNA Plasmid Constructs—The rat IGFBP-3 promoter region in pCAT basic vector (29) was subcloned to plasmids that carry the coding region for firefly luciferase (pGL2-Basic from Promega, Madison, WI). To obtain the −734/+34 pGL2 IGFBP-3, a −1047 to +34 AvaI fragment of the IGFBP-3 promoter in pCAT Basic was blunt-ended, ligated to PUC18, and cut with XbaI/SpeI and subsequently cloned to the XbaI site of pGL2-Basic vector. To obtain the −1600 pGL2 IGFBP-3, we ligated the XbaI/SpeI-cut −734 pGL2 IGFBP-3 to the NheI-XbaI fragment of the −1.6-kilobase pair pCAT Basic vector. To obtain −1601 pGL2 IGFBP-3, we ligated the SpeI-SpeI −1061 to −734 fragment of the −1.06 kilobase pair pGL2 IGFBP-3 in pCAT Basic to XhoI-SpeI-digested −734 pGL2 IGFBP-3. To obtain the −99 pGL2 IGFBP-3, we digested −734 pGL2 IGFBP-3 with Smal and religated the construct. The −1201 pGL2 IGFBP-3 construct was obtained by 5′ deletion of KpnI/MluI-digested −1600 pGL2 IGFBP-3 with exonuclease III.

In order to obtain the IGFBP-3 IRE (−1150 to −1117 bp) constructer, double-stranded oligonucleotides corresponding to the first primer in Table II were annealed, treated with T4 DNA ligase at 16°C for 10 min, and then gel-purified and ligated upstream to a luciferase gene reporter construct (pU40 vector) (pGL3-Promoter from Promega). Orientation of the sequences and the number of tandem repeats were confirmed by dideoxy sequencing.

To obtain the substitution mutants used in Fig. 4, oligonucleotides corresponding to mutants 1–6 (Table II) were used as 5′-primers for 30 cycles of PCR amplification with 3′-primer corresponding to the −503 to −522 bp region of the rat IGFBP-3 promoter. The amplified fragments were subsequently gel-purified, cut with SpeI, and subcloned into the Smal-SpeI-digested −1201/+34 pGL2 IGFBP-3. The presence of mutated bases was confirmed by dideoxy sequencing.

Nuclear Extracts from Hepatic Nonsympathetic Cells and Rat Liver—Liver extracts were isolated and grown in vitro as described above, and the nuclear extracts were prepared by pooling cells from 10–20 mm plates, using the protocol described by Dignam et al. (30) with slight modifications. Aprotinin and leupetin at 2 μg/ml, and pepstatin A at 1 μg/ml were added to 100 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol. The cells were exposed to medium with or without 10−6 M insulin for 48 h prior to nuclear protein extraction on day 5 of culture. Liver nuclear extracts from normal and streptozotocin-diabetic rats were generous gifts of Dr. Ching-I Pao, and were obtained as described previously (31).

DNase I Protection Assay, Gel Mobility Shift Assay, and Southwestern Blotting—For DNase I footprinting, end-labeled 140-bp Smal/SalI fragments corresponding to the −1201 to −1061 bp region of rIGFBP-3 was incubated with 5–20 μg of nuclear extract protein in 25 μl of binding buffer containing 20 mM Hepes, pH 7.9, 0.2 mM EDTA, 0.1 μM KCl, 0.5 mM dithiothreitol, 1 μg of poly(dI-dC) and 20% glycerol at 25°C for 20 min. DNase I at 5 μg/ml in 25 mM NaCl, 10 mM Hepes, 5 mM MgCl2, and 1 mM CaCl2 was then added, followed by incubation at 25°C for 2 min. The reaction was stopped with buffer containing 12.5 mM EDTA, 12.5 μg/ml proteinase K, 10 μg/ml yeast tRNA, and 0.1% SDS at 37°C for 10 min, followed by phenol/chloroform extraction, precipitation with ethanol, and electrophoresis on an 8% urea, 6% polyacrylamide gel.

For gel mobility shift assay, [γ-32P]ATP-labeled oligonucleotides (−1150 to −1117 bp fragment of rat IGFBP-3) was incubated with different concentrations of nuclear extracts in 25 μl of binding buffer containing 10 μg Tris, pH 7.5, 10 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 0.2% Nonidet P-40, 20 μg of bovine serum albumin, 36 μg of salmon sperm DNA, and 10% glycerol at 25°C for 20 min. Incubations were carried out with or without unlabeled competitors as indicated. Protein-DNA complexes were separated from free probe on 6% polyacrylamide gel in 0.25 × TBE at 12 V/cm for 2–3 h, and visualized by autoradiography.

For Southwestern blotting, 20 μg/lane of nuclear extracts from cultured cells treated or untreated with 10−6 M insulin and from pooled liver extracts from normal and diabetic rats were subjected to SDS-polyacrylamide gel electrophoresis, blotted to nitrocellulose, and denatured by incubating in a solution containing 6 M guanidine HCl, 25 mM Hepes, pH 7.6, 12.5 mM MgCl2, 20% glycerol, and 10% sodium dodecyl sulfate at 37°C for 10 min, followed by phenol/chloroform extraction, precipitation with ethanol, and electrophoresis on an 8% urea, 6% polyacrylamide gel.

Statistical Analysis—Data were examined by analysis of variance and Duncan’s multiple range test. Results were considered significant when p was <0.05.

RESULTS

Effect of Insulin on Transcription of IGFBP-3 5′-Deletion Mutants—To investigate the sequences required for insulin to increase rat IGFBP-3 gene transcription, we developed a series of 5′-deletions of the promoter region between nt −1600 and +34. All plasmids were constructed with a common 3′-end (nt +34), attached to a luciferase reporter gene; this design preserves the TATA element (−28) and a CpG island, allowing function as a basal promoter (29). Fig. 1 shows insulin-stimulated expression after transient transfection of hepatic nonparenchymal cells in primary culture. Using plasmids with nested deletions from −1600 to −99 nt, insulin increased luciferase activity 168 ± 36% and 166 ± 15% with IGFBP-3 plasmids that spanned −1600/+34 and −1201/+34 nt, respectively, compared with untreated cells. Insulin had no effect on luciferase activity with plasmids that spanned −1150/+34 and −734/+34 nt. However, insulin increased luciferase activity 58 ± 2% with plasmids containing the −99/+34 nt, presumably representing effects on the basal promoter. These results indicate that the deletion of bases −1201 to −1061 largely eliminated the ability of insulin to stimulate IGFBP-3 expression in liver cells, suggesting that an insulin-responsive element is located within this 140-bp region.
To determine whether this IGFBP-3 sequence permitted a 92% increase in reporter activity driven by the viral promoter, a concatemer was inserted upstream from the SV40 promoter in a luciferase reporter construct, and transfected into hepatic nonparenchymal cells. As shown in Fig. 3, luciferase activity driven by the viral promoter alone was increased by 13 ± 5% in response to insulin, but the presence of the 34-bp IGFBP-3 sequence permitted a 92 ± 12% increase in reporter activity in response to insulin. When the IGFBP sequence was inserted in reverse orientation, insulin increased luciferase expression 91 ± 12%. Thus, the insulin responsiveness of the −1150 to −1117 bp region of the IGFBP-3 gene is portable to a heterologous promoter, and the hormone responsiveness is independent of orientation. In combination, these findings indicate that the IGFBP-3 region functions as an enhancer and is sufficient for regulation by insulin.

Linker-scanning Mutations Identify an IRE in the IGFBP-3 Gene—To determine the minimal IGFBP-3 sequence required for regulation by insulin. An end-labeled 140-bp Smal/Sall (−1201/−1061) fragment of rIGFBP-3 was incubated with nuclear extracts from insulin-treated nonparenchymal cells at 25°C for 20 min and then digested with DNase I. Protected regions were determined according to Maxam-Gilbert sequencing as indicated on the right. The G + A ladders of the fragment on the left were used to read the sequences, and the DNA digested with DNase I (zero nuclear extract) served as negative control. The noncoding strand is shown above, and similar protected regions were noted on the coding strand.

**TABLE I**

Comparison of IGFBP-3 protected regions with known IREs

| IRE          | Glyceraldehyde-3-phosphate dehydrogenase |
|--------------|------------------------------------------|
| GAPDH        | AA CTITCCCGCC TICTCAAGCTT TGAAAG         |
| Glucagon     | GT TTTCACGCC TGACTGAGAT TGAAAGG          |
| Amylase      | CAGTGATTTGCG TGAAGTTTC TAAAGA           |
| IGFBP-1      | GGTGTTTGGAC AGT                         |
| PEPCK        | TG GTGTTTGGAC AAC                       |
| Prolactin    | ATCTACCCGTC ATTAAAGTA                   |
| IGFBP-3      | AATTCAAGGG TATCCAGGAA AGTTCCTTC TAAG    |

This concatemer was inserted upstream from the SV40 promoter in a luciferase reporter construct, and transfected into hepatic nonparenchymal cells. As shown in Fig. 3, luciferase activity driven by the viral promoter alone was increased by 13 ± 5% in response to insulin, but the presence of the 34-bp IGFBP-3 sequence permitted a 92 ± 12% increase in reporter activity in response to insulin. When the IGFBP sequence was inserted in reverse orientation, insulin increased luciferase expression 91 ± 12%. Thus, the insulin responsiveness of the −1150 to −1117 bp region of the IGFBP-3 gene is portable to a heterologous promoter, and the hormone responsiveness is independent of orientation. In combination, these findings indicate that the IGFBP-3 region functions as an enhancer and is sufficient for regulation by insulin.
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parenchymal cells. Table II shows the wild type IGFBP-3 IRE sequence (−1150 to −1117 bp) and the internal substitution mutants used in these studies. Fig. 4 shows the increase in expression in response to insulin in cells transfected with these constructs. In the absence of insulin, all of the constructs had basal activity that varied only slightly above or below the level seen with the control plasmid (wild type −1201/+34 bp). Substitutions of nt −1148/−1144 and −1143/−1139 (mutants 1 and 2) reduced the response to insulin to 3 ± 9% and 23 ± 14% of that of the wild type control plasmid. Mutations of −1138/−1134, −1133/−1129, and −1128/−1124 bases (mutants 3, 4, and 5) reduced the insulin response to 60 ± 32, 61 ± 5, and 47 ± 18% of the control plasmid, respectively. In contrast, mutations of −1123/−1119 (mutant 6) and −1150 to −1150 bp (not shown) had no effects on insulin responsiveness. Thus, mutation of the region from −1148 to −1139 bp reduced the response to insulin by 80–90%, whereas mutation of the −1138 to −1124 bp region reduced the response to insulin by 50–60%, while mutation in other neighboring regions had little effect. These data indicate that the region between −1148 and −1139 bp is necessary for insulin responsiveness of IGFBP-3 gene transcription.

DNA-Protein Interactions with the IGFBP-3 IRE—To characterize the interactions of nuclear factors with the IGFBP-3 IRE, we examined binding activity by gel mobility shift analysis. End-labeled −1150 to −1117 bp oligonucleotides were incubated with nuclear extracts from hepatic nonparenchymal cells and subjected to nondenaturing polyacrylamide gel electrophoresis. As shown in Fig. 5A, one major DNA-protein complex was observed consistently, with a second band of higher mobility that was present intermittently. The binding activity of nuclear extracts obtained from insulin-treated cells was about 3-fold higher than that of extracts from control cells. Fig. 5B demonstrates the specificity of complex formation. The formation of the major DNA-protein complex was inhibited by mutant 1 of the −1148 to −1117 bp region (not shown) had no effects on insulin responsiveness. Thus, mutation of the region from −1148 to −1139 bp reduced the response to insulin by 80–90%, whereas mutation of the −1138 to −1124 bp region reduced the response to insulin by 50–60%.

Fig. 3. Effects of insulin on the expression of −1150 to −1117 bp rat IGFBP-3 in heterologous promoter. The pGL3 promoter vector (which contains an SV40 promoter/luciferase reporter gene insert but not the SV40 enhancer) was used to construct hybrid DNA with three tandem copies of the −1150 to −1117 bp region of IGFBP-3 promoter. Nonparenchymal cells were transfected with 2.5 μg of the plasmids containing the concatemer of the −1150−1117 bp region upstream of the SV40 promoter in 5′→3′ orientation (−1150/−1117 SV40 promoter) or in 3′→5′ orientation (−1150/−1117 R SV40 promoter). The plasmid without the IGFBP-3 region (SV40 promoter) served as control. After transfection, insulin at 10−6 M was added to half of the cultures, and the luciferase activity was measured 24 h later. The percentage increase in luciferase activity of the constructs in the presence of insulin compared with the activity in the absence of insulin is shown after subtracting the background activity of the vector plasmid. Background activity of the SV40 promoter construct was 24.5 ± 3 arbitrary light units, basal activity of the −1150/−1117 SV40 promoter was 510−650 units, and insulin increased the activity to 950−1030 units (similar ranges were noted with the 3′→5′ SV40 promoter construct). Results from two separate experiments with four samples each are shown above as mean ± S.E. Similar results were obtained in three experiments.

Table II

| Oligonucleotides   | Sequence               |
|--------------------|------------------------|
| IGFBP-3 IRE        | −1150 AATTCAGGGTATCCAGGAAAGTCTCTTCTTAAG −1117 |
| Mutant 1           | AAacctggGTTATCCAGGAAAGTCTCTTCTTAAG |
| Mutant 2           | AATTCAGAaagGTCAGGAAAGTCTCTTCTTAAG |
| Mutant 3           | AATTCAGGATTTGGAAGTCTCTTCTTAAG |
| Mutant 4           | AATTCAGGATTTGGATCCAGGAAGTTCTCTCTTAAG |
| Mutant 5           | AATTCAGGGTATCCAGGAAAGTCTTTTCTTAAG |
| Mutant 6           | AATTCAGGGTATCCAGGAAAGTCTCCTCtcqAG |

Sequences are numbered relative to transcription initiation site of rat IGFBP-3 gene. Underlined bases (lowercase type) are mutated.

To identify the nucleotide sequences within the 34-bp region that were necessary for complex formation, we generated a series of oligonucleotides with 5-bp substitution mutations within the −1150 to −1117 bp fragment (as shown in Table II). Competition assays were conducted using a wild type fragment as a probe, and an excess of unlabeled mutant oligonucleotides as competitors. As shown in Fig. 7, the DNA-protein complexes were competed away in the presence of a 100-fold excess of unlabeled wild type fragment. When the mutants were used as competitors, the oligonucleotides with substitution of sequences corresponding to nt −1148/−1144 (mutant 1) and −1143/−1139 (mutant 2) could not compete away the DNA-protein complexes (band intensity was comparable with that seen with a 100-fold excess of unrelated oligonucleotides shown in Fig. 5B). However, mutants with substitution of sequences from −1138 to −1119 (mutants 3–5) were able to compete for binding of nuclear protein(s) to the IGFBP-3 IRE. These results suggest that the sequences between −1148 and −1139 are essential for binding of the nuclear factor(s) to the IGFBP-3 IRE. Thus, the same 10-bp region that is critical for insulin responsiveness in functional studies is also important for binding of putative trans-acting factor(s).
Fig. 4. Effect of substitution mutations of nt −1148 to −1119 on insulin stimulation of the rat IGFBP-3 promoter. Substitution mutants were prepared from 5′-primers shown in Table II and described under “Experimental Procedures.” All of the mutants have IGFBP-3 promoter sequences starting from position −1201 and extending to +34, but with 5-bp substitutions inserted at various positions within the −1148 to −1119 region. Transfection into nonparenchymal cells and luciferase assay of the lysate are as described in Fig. 1. The increase in IGFBP-3 luciferase expression in response to insulin of the control plasmid was expressed as 100%, and the response to insulin of the mutants was expressed relative to the response of the control plasmid. These results are from three experiments that were performed in triplicate and expressed as mean ± S.E.

To characterize the size and hormone responsiveness of proteins associated with the IGFBP-3 IRE, nuclear extracts from hepatic nonparenchymal cells and rat livers were subjected to SDS-polyacrylamide gel electrophoresis, blotted to nitrocellulose, and probed with a labeled IGFBP-3 IRE oligonucleotide, as shown in Fig. 8. Proteins with apparent molecular mass of 90 and 70 kDa were present in both cell and liver extracts. Metabolic responsiveness of the 70-kDa protein was not consistent with different preparations of nuclear extracts, while the 90-kDa protein appeared to be hormone-responsive. The abundance of the 90-kDa protein was increased 2–2.5-fold with insulin treatment in cultured cells and was increased 1.8-fold in hepatic extracts from normal compared with diabetic animals.

Discussion

Our previous studies demonstrated that the Kupffer and sinusoidal endothelial cells of the liver are the locus of both high basal expression of the IGFBP-3 gene and transcriptional regulation of IGFBP-3 by insulin (27, 28). In this investigation, we used transient transfection of these nonparenchymal cells to identify sequences within the −1150 to −1124 bp region of the IGFBP-3 promoter that are required for the response to insulin. The 26-bp region appears to be both sufficient and necessary for insulin responsiveness, since this region can function as an IRE in the context of a heterologous promoter, and mutations of these bases eliminate the insulin response of the parent plasmid. The IGFBP-3 basal promoter (−99 to +34 bp) also exhibits modest responsiveness to insulin, presumably reflecting involvement of factors that are part of the basal transcriptional machinery, as has been described previously (37). Our mutational analysis defines a 10-bp core sequence between −1148 and −1139 bp that is critical for the effects of insulin, but full responsiveness appears to require the adjacent 16 bp.

The IGFBP-3 IRE appears to be composed of a dyad of AGG(A/G)A. This sequence has a strong resemblance to the recognition sequence of Ets-related transcription factors, AGGAA, which is contained within the insulin response elements of both the prolactin and somatostatin genes (38). The imperfect dyad suggests the possibility of a dimer binding motif, and Ets-related proteins tend to function most effectively from dyad recognition sites (39). In addition to the above cognate binding sequence, −1134 to −1123 bp of the IGFBP-3 IRE (GGAAAGTCTCC on the sense strand) strongly resembles the binding sites for HIV-xB proteins, which recognize GGAAGTCC, and NF-κB proteins, which recognize GGAAGTCC (40). However, studies with antibodies to two subunits of NF-κB (c-Rel, and P50) did not alter the DNA-protein complexes as seen in Fig. 5 (not shown). The 10-bp core sequence of the IGFBP-3 IRE that is most critical for insulin responses (−1148/−1139) had no significant consensus sequence similarity to previously identified transcription factor binding sites. We are currently in the process of evaluating binding by these and other factors.

When the minimal sequence of the IGFBP-3 IRE is compared with the sequence of other genes that are regulated by insulin, there are weak homologies with the IREs of several genes, including prolactin, glucagon, and amylase. As shown in Table I, the 5′-end of the IGFBP-3 IRE exhibits somewhat more
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homology with IREs identified for glucagon and prolactin, while the 3'-end of the IRE appears to have more homology with the IREs from amylase and PEPCK. In addition, IGFBP-3 also contains a CAACAAACAATTCC motif (−1159 to −1137 bp) which has some homology to the hepatocyte nuclear factor 3α binding site of IGFBP-1, a related protein which is inhibited by insulin in hepatocytes (41, 42). However, our transfection studies indicate that such a region is not required for regulation of IGFBP-3 transcription by insulin. These limited homologies between the IGFBP-3 IRE and other known IREs suggest the possibility that some common factor(s) may regulate the expression of genes that are responsive to insulin.

Use of the IGFBP-3 IRE (−1150 to −1117 bp) as a probe in gel mobility shift analyses revealed a single insulin-inducible DNA-protein complex with nuclear extracts from nonparenchymal cells. Similar insulin-responsive up-regulation of protein binding has been reported for upstream fatty acid synthase sequences in hepatocytes, and for the prolactin gene in GH₄ cells (38, 43). In contrast, the presence of insulin does not affect DNA-protein interactions with the PEPCK and IGFBP-1 genes. Such a difference may reflect the action of insulin on gene transcription via direct or indirect mechanisms. Insulin stimulates gene activity with increased binding of upstream stimulatory factor 1 to fatty acid synthase (43) and Ets-related proteins to the prolactin insulin response region (38) as opposed to the negative effects of insulin on PEPCK and IGFBP-1 gene transcription. Insulin is postulated to mediate such negative effects indirectly, by inhibiting hepatocyte nuclear factor 3 binding that supports glucocorticoid-induced transcription of the gene (41, 42). Our findings are consistent with a model in which insulin induces factors that bind to the IGFBP-3 IRE and increase IGFBP-3 gene transcription directly. Since insulin deprivation in vivo in streptozotocin-diabetic rats reduced DNA-protein complex formation with the IGFBP-3 IRE in a manner similar to that seen with insulin deprivation of hepatic nonparenchymal cells in primary culture, the results in combination support the physiologic relevance of this insulin-responsive DNA-protein complex in mediating the action of insulin on the transcription of the IGFBP-3 gene.

Detailed analysis of the sequences critical to formation of DNA-protein complexes with the IGFBP-3 IRE revealed that the retarded band represents contact of factors with the 10-bp core sequence between −1148 and −1139 bp. (a) Oligonucleotides mutated in this region compete poorly with the labeled wild type probe. (b) When such mutant oligonucleotides are used as probes, no DNA-protein complexes were found (data not shown). In addition, (c) use of −1157 to −1139 bp oligonucleotides as probes (including the 10-bp core sequence but lacking the adjacent 16-bp region between −1138 and −1124 bp) revealed bands of mobility similar to those seen with the −1150 to −1117 bp probes; however, apparent affinity of DNA-protein binding was considerably lower, suggesting that other proteins or DNA contact of proteins with the −1138 to −1129 bp region may act synergistically with a contiguous 5'-region to enhance transcription factor binding. This indicates that there is no simple one-to-one correspondence between the IRE DNA-binding motifs and the DNA/protein interface; instead, the adjacent bases are critical for site-specific recognition (44). This is consistent with the major DNA binding domain requiring neighboring regions to make folding and docking of the protein possible. Alternatively, multiple DNA binding domains may be required for complete site-specific recognition, as could occur if the binding protein is a homodimer or heterodimer or if the binding protein contains different motifs in the same complex.

Our Southwestern blotting studies with IGFBP-3 IRE probes indicate the presence of a 90-kDa insulin-responsive DNA-binding protein that can be detected both in hepatic nonparenchymal cells in primary culture and in rat liver. The 90-kDa band was consistently increased by the addition of insulin to the cultured cell system and decreased by the induction of insulin deficiency in streptozotocin-treated rats. In contrast,
the 70-kDa protein did not exhibit consistent responsiveness to metabolic status. Both proteins appear to bind directly to the core sequence of the –1148 to –1139 bp core sequence, since similar bands were observed with –1157 to –1139 bp and –1150 to –1117 bp probes. Thus, both mutational analysis of IGFBP-3 function in cell transfection models and studies of DNA-protein interactions in both gel shifts and Southwestern blotting point to an alteration in the quantity and/or activity of factors binding to the –1148 to –1139 bp region as a critical mechanism for the control of IGFBP-3 transcription by insulin.

Our studies add to the understanding of the mechanism through which IGFBP-3 expression is regulated by insulin. We also demonstrate the feasibility of transient transfection in hepatic nonparenchymal cells in primary culture, which should be useful for other studies of genes that are uniquely expressed by these cells. Finally, our identification of an IRE in the IGFBP-3 gene and its association with hormone-responsive IRE binding proteins should provide insights important for future identification of the factors involved. Since IGFBP-3 is the major carrier protein for IGF-I, a critical growth factor, our observations may also shed light on the processes through which poor metabolic control of diabetes mellitus leads to impaired growth.

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