Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 226-232

INSULIN AND GLUCOCORTICOIDS REGULATE IGFBP-1 EXPRESSION VIA A COMMON PROMOTER REGION

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Received January 25, 1994

Summary: Hepatic expression of insulin-like growth factor binding protein-1 is regulated by insulin and glucocorticoids. To study underlying mechanisms, rat hepatocytes in primary culture were transfected with deletion mutants and heterologous promoter constructs, identifying a 41 bp region of the rat insulin-like growth factor binding protein-1 promoter which is sufficient to mediate regulation by both insulin and glucocorticoids. Half maximal suppression of promoter activity by insulin occurred at a physiologic concentration, 5×10^{-10} M, and regulation by insulin was dominant in that insulin suppressed promoter activity at all dexamethasone concentrations. Transfection of rat hepatocytes in primary culture should be a useful approach for exploring the regulation of gene expression by insulin.

The liver is the major source of circulating IGFBP-1. IGFBP-1 accounts for 2 to 3% of the serum IGF binding activity, and may modulate "free" insulin-like growth factor-I levels (1). Serum levels of this 26 kDa protein rise in conditions of malnutrition or diabetes (2,3). Multiple hormones, including glucocorticoids, glucagon and growth hormone, regulate hepatic production of IGFBP-1, but the suppressive effects of insulin on gene expression are dominant (2,4,5). Insulin regulates IGFBP-1 expression at the level of transcription: in primary cultures of rat hepatocytes, insulin suppresses IGFBP-1 mRNA levels and gene transcription rates by more than 80% (4,6). In addition, a recent report suggests that IGFBP-1 may be a marker of hepatic insulin

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ABBREVIATIONS USED: **IGFBP-1**, insulin-like growth factor binding protein 1; **CAT**, chloramphenicol acetytransferase; **IRR**, insulin responsive region; **SV40**, simian virus 40; **GRE**, glucocorticoid response element; **IRE**, insulin response element; **IDE**, insulin dependent element; **PEPCK**, phospho*enol*pyruvate carboxykinase.

0006-291X/94 $5.00

sensitivity (7). Therefore, we chose to evaluate the regulation of the IGFBP-1 promoter by insulin and glucocorticoids in primary cultures of rat hepatocytes.

Recent studies have evaluated regulation of the human IGFBP-1 promoter by insulin in hepatoma cells (8), but regulation of promoter activity in normal hepatocytes has not been examined. Furthermore, although hepatocyte production of IGFBP-1 is dependent on the presence of glucocorticoids (4), the location of the glucocorticoid response element in the IGFBP-1 promoter has not been previously reported. In the following studies, we identify a region of the rat IGFBP-1 promoter which is sufficient for regulation of promoter activity by both insulin and glucocorticoids.

METHODS: IGFBP-1 promoter/CAT reporter gene constructs were prepared either by standard restriction digestion and ligation of genomic rat IGFBP-1 fragments into the pCAT-Basic plasmid (Promega, Madison, WI), or by reinsertion of a fragment of rat genomic DNA (ligated to the CAT coding region and SV40 regions of the pCAT-Basic) into pGEM-3Z (Promega), the parent plasmid of pCAT-Basic. For all IGFBP-1/CAT constructs, the 3' end of the rat genomic sequence was the *Sfa*N I site at +148 bp. For heterologous promoter constructs, a 44 bp fragment, containing 3 bp of polylinker and 41 bp (-118 to -78) of rat genomic sequence, was removed from p-114BPCAT by *Bam*H I digestion and ligated into pCAT-Promoter (Promega) at the *Bgl* I site 5' to the SV40 promoter. All plasmid DNA used in the transfection studies was purified twice by cesium chloride gradient centrifugation (9). The pRSVLUC plasmid was kindly provided by Dr. D.R. Helinski (10).

Hepatocytes were isolated from 150 to 200 g male Sprague-Dawley (Harlan, Indianapolis, IN) rats by a modification of the collagenase (Worthington, Freehold, NJ) perfusion method of Seglen, as previously reported (4). Hepatocytes were plated on collagen-coated 60 mm plates at a density of 5×10^6 cells per plate in DMEM/F12 medium (Mediatech, Herndon, VA) with 10% Custom Blend 995 serum (Hyclone, Logan, UT). Non-adherent cells were removed after 30 min and the medium replaced with 3 ml defined medium (DMEM/F12 containing 1 mg/ml bovine serum albumin, and 0.1 mg/ml transferrin) plus 10^{-7} M insulin, dexamethasone per experimental protocol, and 3% serum. Cells were transfected overnight with 6 μg of DNA-calcium phosphate precipitate (5 μg of test construct +1 μg pRSVLUC) applied 5 h after plating (11). On Day 2, the cells were rinsed twice with 4 ml phosphate-buffered saline and placed in 3 ml of serum-free defined media containing stated concentrations of insulin and dexamethasone. After 24 h, the cells were rinsed twice with 4 ml phosphate buffered saline and scraped into 1 ml of 40 mM Tris, 0.15 M NaCl, 1 mM EDTA pH 7.4. Cells were pelleted, then resuspended in 200 μl of 0.25 M Tris pH 7.5 and a 40 μl aliquot was transferred to a tube containing 40 μl of 1x Promega cell lysis buffer supplemented with 0.5% Triton X-100, and 2 mg/ml bovine serum albumin. The remaining cell suspension was lysed by thee cycles of freeze-thaw. The Promega buffer lysates were assayed for luciferase activity by the Promega Luciferase Assay System protocol in a Microsure 100 luminometer in the manual mode. CAT activity was determined in 75 μl of freeze-thaw lysate, heat-treated at 65°C for 10 min to destroy deacetylase activity (12). The CAT assays contained 0.5 mg/ml bovine serum albumin, 0.25 μCi [^{14}C]-chloramphenicol (Amersham, Arlington Hts, IL), 0.5 mM N-butyryl-coenzyme A, incubated overnight at 37°C, then extracted with 2 volumes of 2:1 2,6,10,14-tetramethylpentadecane:xylenes, with quantitation by liquid scintillation counting of the organic phase. This extraction transferred < 0.1% of the non-acylated

[^{14}C]-chloramphenicol from the aqueous phase (data not shown). Linearity was demonstrated for each luciferase and CAT assay. CAT values were normalized to luciferase values for each lysate, with transfections performed in triplicate plates for each condition.

RESULTS: To identify the insulin responsive region of the IGFBP-1 promoter, a series of deletion mutants were generated. Suppression of CAT activity by insulin was seen with constructs containing 1671, 930, 277 and 118 bp of the IGFBP-1 promoter 5' region (Fig. 1). Further deletion of the promoter to -82 bp (p-82BPCAT) eliminated suppression of CAT activity by 10^{-6} M insulin (Fig. 1). Although normalized CAT activity of p-82BPCAT was reduced 5 to 10 fold versus the p-118BPCAT construct, consistent promoter activity remained, whereas pCAT-Basic (the promoterless parent construct) produced no detectable CAT activity (not shown), consistent with the -82 to +154 bp region functioning as a basal promoter.

In dose response studies, the half maximal inhibitory concentration for insulin (IC_{50}) was approximately 5 x 10^{-10} M (Fig. 2). This concentration is similar to our previously published IC_{50} for suppression of endogenous gene expression by insulin in hepatocytes in primary culture (4), and to nonfasting insulin levels in rats (13). Thus, regulation of the expression of the transfected IGFBP-1/CAT constructs occurred at physiologic insulin concentrations.

To determine if the 37 bp putative IRR between -118 and -82 bp was sufficient for regulation by insulin, the -118 to -78 bp (41 bp) rat IGFBP-1 promoter region was transferred to the heterologous pCAT-Promoter plasmid, which contains an SV40 promoter/CAT reporter insert, but not the SV40 enhancer. The 41 bp IRR was

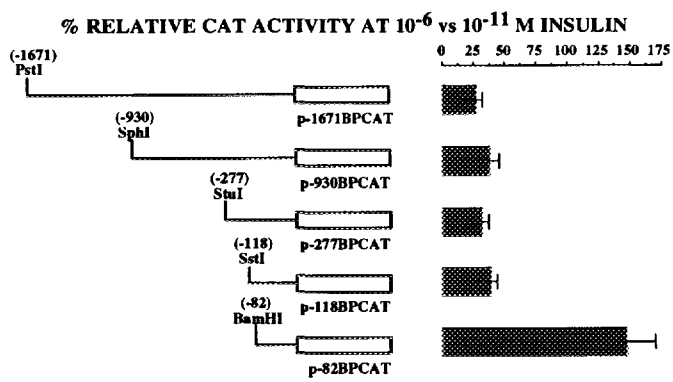
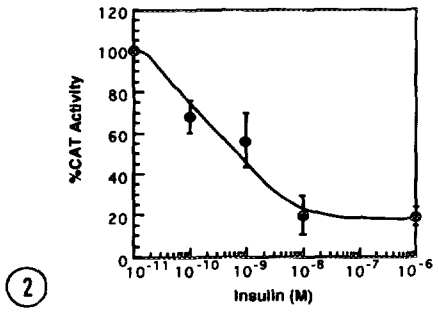
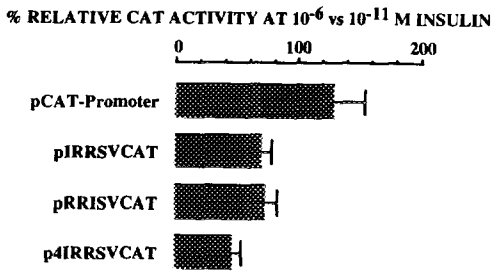


Figure 1. IGFBP-1 promoter CAT reporter constructs were transfected into hepatocytes and then treated with 10^{-6} M or 10^{-11} M insulin for 24 h. The ratios of CAT activities are shown as the mean (±SEM) of 4 to 6 separate transfections. Hepatocytes were treated with 10^{-7} M dexamethasone throughout.



(2)



(3)

Figure 2. Hepatocytes were transfected with p-1671BPCAT then treated with the shown concentrations of insulin for 24 h and 10^{-7} M dexamethasone throughout. CAT activity is expressed as a percent of activity at 10^{-11} M insulin. The means (±SEM) for three separate experiments are shown.

Figure 3. Constructs were transfected into hepatocytes treated with 10^{-6} M or 10^{-11} M insulin for 24 h, and the ratios of CAT activities are shown as the mean (±SEM) for 3 separate transfections. Hepatocytes were treated with 10^{-7} M dexamethasone throughout. To evaluate the impact of the IRR on promoter activity, CAT activity of the heterologous constructs at 10^{-11} M insulin was normalized to that of pCAT-Promoter, and the fold increase in CAT activity was pIRRSVCAT 3.5 ± 0.8; pRRISVCAT 2.7 ± 0.8; and p4IRRSVCAT 16.4 ± 3.1.

inserted upstream (pIRRSVCAT), upstream in an inverted orientation (pRRISVCAT), and upstream as a 4-copy concatemer (p4IRRSVCAT). Suppression of SV40 promoter activity by insulin was consistent, regardless of orientation, and most striking with the 4-copy concatemer construct (p4IRRSVCAT) (Fig. 3). Additionally, the insertion of an IRR element increased the level of CAT expression in transfected cells, compared to the level of expression produced by the parent construct pCAT-Promoter (Fig. 3 legend). This increase in CAT expression, independent of insulin concentration, suggests that factors which enhance IGFBP-1 promoter activity may also act via the 41 bp IRR.

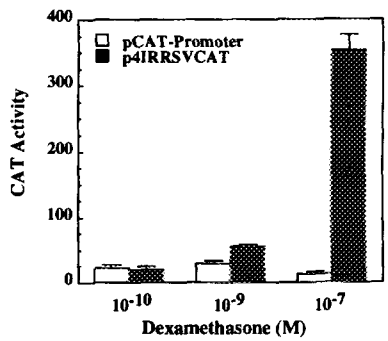


Figure 4. Hepatocytes were placed in the shown concentrations of dexamethasone 30 min after plating and 10^{-11} M insulin for the final 24 h. Mean normalized CAT activities (±SEM) from triplicate plates are shown. The experiment was representative of four.

Glucocorticoids stimulate IGFBP-1 expression (4), and both the deletion analysis and heterologous promoter construct studies we performed suggested that the presence of the -118 to -78 bp promoter region enhanced CAT expression. Therefore, we tested the effect of glucocorticoids on expression of the heterologous promoter construct p4IRRSVCAT in transfected hepatocytes. Glucocorticoids produced an increase in CAT activity over the range of 10^{-10} to 10^{-7} M dexamethasone for p4IRRSVCAT, but not for pCAT-Promoter (Fig. 4). At each tested dexamethasone concentration, suppression of CAT activity by insulin persisted (data not shown). Therefore, the 41 bp region from -118 to -78 bp is sufficient to transfer regulation by both insulin and by glucocorticoids to the SV40 promoter.

DISCUSSION: We have used transfection of IGFBP-1 promoter/CAT reporter constructs into hepatocytes from rats to show that a 41 bp region of the IGFBP-1 promoter contains sequences which are sufficient to mediate both suppression of gene expression by insulin and stimulation of gene expression by glucocorticoids. We also show that the p-82BPCAT construct, which contains the critical HNF-1 binding site at -61 to -49 bp (14-16), possesses basal promoter activity. Having defined an IRR, it is possible to analyze this 41 bp region for homology to other insulin regulated genes and for the presence of transcription factor consensus sequences (Figure 5).

Within the 41 bp IRR, there is a 25/25 bp match between the rat and human IGFBP-1 sequences (-109 to -85 bp in the rat gene and -120 to -96 bp in the human gene) (8,15). Within the 25 bp region, two 8 bp sequences with dyad symmetry are present, and mutation of these sequences has been shown to diminish regulation of the human IGFBP-1 promoter by insulin (8). We have shown that glucocorticoids stimulate IGFBP-1 expression, and therefore the -118 to -78 bp region of the promoter must contain a functional GRE. In the rat gene, just downstream of the 3' end of the

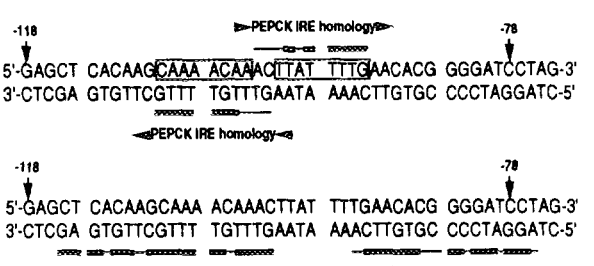


Figure 5. Homology between the rat IGFBP-1 putative IRR and other hormone response sequences: thick lines are homologous nucleotides; thin lines are nonhomologous nucleotides; boxed are two 8 bp sequences with dyad symmetry.

putative insulin responsive element dyad, there is a 10/15 bp match to the GRE consensus sequence (17). The PEPCK promoter also contains the motif of adjacent insulin and glucocorticoid response elements, and the hepatic PEPCK promoter is both suppressed by insulin and stimulated by glucocorticoids (18). However, although the 8 bp insulin responsive sequences of the IGFBP-1 promoter show homology to the PEPCK IRE, the PEPCK gene does not show the same dyad symmetry (18). Thus, insulin may suppress IGFBP-1 and PEPCK promoter activities by different mechanisms.

The 41 bp rat IGFBP-1 IRR also contains a 15/18 bp sequence (-115 to -98 bp) which is homologous to the IDE of the mouse α-amylase gene (19). This 18 bp sequence overlaps with the 5' half of the promoter sequence which shows dyad symmetry, but not with sequences which are homologous to the consensus GRE. However, regulation of α-amylase differs from that of IGFBP-1, in that α-amylase expression is stimulated, not suppressed, by insulin, possibly though decreased binding of a transcription inhibitory factor and increased binding of a tissue specific transcription enhancing factor (18). At this time, the transcription factors which interact with the α-amylase and the PEPCK promoters have not been identified. Elucidation of the mechanism for regulation of IGFBP-1 expression by insulin may provide insights into the regulation of other insulin-sensitive promoters.

In summary, we have used both deletion analysis and heterologous promoter constructs to identify a 41 bp region of the IGFBP-1 promoter which is sufficient to mediate regulation by both insulin and glucocorticoids. Transfection of rat hepatocytes in primary culture should be a useful approach for future studies exploring the regulation of gene expression by insulin.

ACKNOWLEDGMENTS: This work was supported in part by NIH Grants DK-08147 (to DGR); DK-07298; DK-08978 (to PMT), grants from the Emory Medical Care Foundation and the Emory University Research fund to DGR, and from the MRC of Canada to LJM. We would like to thank Chis Noel for technical assistance, and Mary Lou Mojonnier and Sharon DePeaza for assistance in preparing this manuscript.

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