A Multicomponent Insulin Response Sequence Mediates a Strong Repression of Mouse Glucose-6-phosphatase Gene Transcription by Insulin*

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Glucose-6-phosphatase (G6Pase) catalyzes the final step in the gluconeogenic and glycogenolytic pathways. The transcription of the gene encoding the catalytic subunit of G6Pase is stimulated by glucocorticoids, whereas insulin strongly inhibits both basal G6Pase gene transcription and the stimulatory effect of glucocorticoids. To identify the insulin response sequence (IRS) in the G6Pase promoter through which insulin mediates its action, we have analyzed the effect of insulin on the basal expression of mouse G6Pase-chloramphenicol acetyltransferase (CAT) fusion genes transiently expressed in hepatoma cells. Deletion of the G6Pase promoter sequence between −271 and −199 partially reduces the inhibitory effect of insulin, whereas deletion of additional sequence between −198 and −159 completely abolishes the insulin response. The presence of this multicomponent IRS may explain why insulin potently inhibits basal G6Pase-CAT expression. The G6Pase promoter region between −198 and −159 contains an IRS, since it can confer an inhibitory effect of insulin on the expression of a heterologous fusion gene. This region contains three copies of the T(IGA)TTTGT sequence, which is the core motif of the phosphoenolpyruvate carboxykinase (PEPCK) gene IRS. This suggests that a coordinate increase in both G6Pase and PEPCK gene transcription is likely to contribute to the increased hepatic glucose production characteristic of patients with non-insulin-dependent diabetes mellitus.

While insulin has long been known to modulate intracellular metabolism by altering the activity or intracellular location of various enzymes, it is only more recently that the regulation of gene transcription by insulin has been recognized as a major action of this hormone (1). cis-Acting elements that mediate the action of insulin on gene transcription, referred to as an insulin response sequence or elements (IRSs/IRESs),¹ have been identified in a number of genes but, unlike cAMP (2, 3), which regulates gene transcription predominantly through one cis-acting element, it is already apparent that a single consensus IRS does not exist (1). Instead, most of the sequences identified to date appear unique, a situation that resembles that for phorbol esters that regulate gene transcription through at least eight distinct consensus sequences (4).

Non-insulin-dependent diabetes mellitus (NIDDM) is characterized by defects in insulin secretion, peripheral glucose utilization, and hepatic glucose production (HGP) (5). The ability of insulin to stimulate peripheral glucose utilization and repress HGP in patients with NIDDM is reduced, a phenomenon known as insulin resistance (5). Various investigators have speculated that an alteration in the insulin-regulated expression of specific genes, a consequence of insulin resistance, may contribute to the pathophysiology of this disease (1, 5, 6). The gene encoding the glucose-6-phosphatase (G6Pase) catalytic subunit (7) is one such candidate, since it catalyzes the final step in the gluconeogenic pathway, the conversion of glucose-6-phosphate to glucose (8). The activity (9, 10) and mRNA level (10–12) of the G6Pase catalytic subunit is increased in diabetic animals, and this contributes to increased fasting HGP (13).

Experiments in FAO rat hepatoma cells (14) and primary hepatocytes (15) have shown that insulin treatment reduces both basal and glucocorticoid-induced G6Pase mRNA levels. Since the increased fasting HGP characteristic of NIDDM may be explained, in part, by an increase in G6Pase gene expression resulting from a loss of insulin repression, it is of interest to determine the mechanism whereby insulin normally inhibits G6Pase gene expression. This manuscript describes the identification of a potent IRS in the G6Pase promoter.

EXPERIMENTAL PROCEDURES

Materials—[α-³²P]dATP (3000 Ci mmol⁻¹) and [³H]Acetate, sodium salt (>1 Ci mmol⁻¹) were obtained from Amersham Corp. and ICN, respectively. Insulin was purchased from Collaborative Bioproducts. All other chemicals were of the highest grade available.

Cloning of the Mouse G6Pase Gene and Promoter—DNA manipulations were accomplished by standard techniques (16). A mouse liver λ genomic library (128SV, Lambda FIX II vector) (Stratagene) was screened with a probe encoding the rat glucose-6-phosphatase cDNA open reading frame (EcoRI/HindIII fragment) (12). DNA from positive clones was digested with NotI and EcoRI, and DNA restriction fragments were separated by agarose gel electrophoresis, transferred to a nitrocellulose membrane, and sequencing hybridized with the rat glucose-6-phosphatase open reading frame cDNA probe and an oligonucleotide spanning nucleotides 25–47 of exon 1 of the published genomic sequence. This region corresponds to the nucleotides spanning nucleotides 25–47 of exon 1 of the published genomic sequence. This region contains three copies of the T(IGA)TTTGT sequence, which is the core motif of the phosphoenolpyruvate carboxykinase (PEPCK) gene IRS. This suggests that a coordinate increase in both G6Pase and PEPCK gene transcription is likely to contribute to the increased hepatic glucose production characteristic of patients with non-insulin-dependent diabetes mellitus.

¹ The abbreviations used are: IRS, insulin response sequence; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; IGFBP-1, insulin-like growth factor-binding protein-1; TAT, tyrosine aminotransferase; ApoCIII, apolipoprotein CIII; NIDDM, non-insulin-dependent diabetes mellitus; HGP, hepatic glucose production; CAT, chloramphenicol acetyltransferase; bp, base pair(s); TK, thymidine kinase.

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cells were grown to 40–70% confluence in T150 flasks in
G6Pase-CAT fusion gene expression. H4IIE cells were transiently co-transfected, as described previously (20), with either a G6Pase-CAT or a PEPCK-CAT fusion gene (15 μg) containing promoter sequence from −751 to +66 or −486 to +69, respectively, and an expression vector (5 μg) encoding the glucocorticoid receptor. Following transfection, cells were incubated for 18–20 h in serum-free medium in the absence of hormones (C) or in the presence of various combinations of 10 nM insulin (I) and 500 nM dexamethasone (D) (panel A) or (panel B) in the presence (D) or absence (C) of 10 nM insulin (panel B). The cells were then harvested and CAT activity assayed as described under “Experimental Procedures.” In A results are presented as the ratio of CAT activity in dexamethasone- or dexamethasone plus insulin-treated cells relative to control cells (expressed as fold induction). In panel B results are presented as the ratio of CAT activities in insulin-treated versus control cells (expressed as percent of control). The data represent the mean of three to four experiments ± S.E.

Plasmid Construction—The orientation of all subcloned DNA fragments was determined by restriction enzyme analysis and confirmed by DNA sequencing using the U. S. Biochemical Corp. Sequenase kit. A fragment of the mouse G6Pase promoter from −751 to +66, relative to the transcription start site, was ligated into the polylinker of the pCA-T(An) expression vector, a generous gift from Dr. Howard Towle (18). A series of truncated G6Pase-CAT fusion genes was generated by either restriction enzyme digestion or polymerase chain reaction, using standard techniques (16), with the 5′ end points shown in Fig. 2. All promoter fragments generated by polymerase chain reaction were completely sequenced to ensure the absence of polymerase errors, whereas promoters generated by restriction enzyme digestion were only sequenced to confirm the 5′ end points.

Plasmid TKC-VI, kindly provided by Dr. Thomas Sudhof (19), contains the herpes simplex virus thymidine kinase (TK) promoter ligated to the CAT gene. The TK promoter sequence extends from −480 to +51 and contains a BamHI linker between positions −40 and −35 (Fig. 3B). Various double-stranded oligonucleotides representing the G6Pase promoter sequence between −197 and −159 (Fig. 3A) were synthesized with BamHI-compatible ends using a Perceptive Biosystems Nucleic Acid Synthesis System and were cloned in either orientation into BamHI-cleaved TKC-VI by standard techniques (16). All plasmid constructs were purified by centrifugation twice through cesium chloride gradients (16).

Cell Culture and Transient Transfection—(a) Rat H4IIE hepatoma cells were grown to 40–70% confluence in T150 flasks in α-modified Eagle’s medium containing 2% (v/v) fetal calf serum, 3% (v/v) newborn calf serum, and 5% (v/v) calf serum and were then transiently transfected in solution using the calcium phosphate-DNA co-precipitation method as described previously (20). Expression vectors encoding β-galactosidase (2.5 μg) and/or the insulin receptor (5 μg), a generous gift from Dr. Jonathan Whittaker (23), were co-transfected with the reporter gene construct (15 μg).

(b) Human HepG2 cells were grown to 40–70% confluence in T150 flasks in Dulbecco’s modified Eagle’s medium containing 2.5% (v/v) newborn calf serum and 2.5% (v/v) fetal calf serum and 5% (v/v) NuSerum IV (Collaborative Research, Inc.) and were then transiently transfected in solution using the calcium phosphate-DNA co-precipitation method as described previously (22). Expression vectors encoding β-galactosidase (2.5 μg) and/or the insulin receptor (5 μg), a generous gift from Dr. Jonathan Whittaker (23), were co-transfected with the reporter gene construct (15 μg).

CAT and β-Galactosidase Assays—CAT and β-galactosidase assays were performed exactly as described previously (24). Since β-galactosi-
dase is very poorly expressed in H4IIE cells (20, 22), CAT activity was corrected for the protein concentration in the cell lysate, as measured by the Pierce BCA assay, and each plasmid construct was transfected multiple times. CAT activity expressed in HepG2 cells was either corrected for the protein concentration in the cell lysate or β-galactosidase activity. Both methods gave similar results (Fig. 2B).

RESULTS AND DISCUSSION

Insulin Represses Dexamethasone-stimulated G6Pase-CAT and PEPCK-CAT Fusion Gene Expression—To study the molecular mechanism whereby insulin inhibits G6Pase gene expression, the promoter of the mouse G6Pase gene was isolated (see “Experimental Procedures”). The start site for mouse G6Pase gene transcription, previously reported by Shelly et al. (17), is 1 bp 3′ of that reported for the human gene (25) but 5 bp 5′ of that reported for the rat G6Pase gene (15).

A fragment of the mouse G6Pase promoter from −751 to +66, relative to the transcription start site was ligated into the pCAT(An) expression vector (18) and the resulting construct transiently transfected into H4IIE cells. Fig. 1A shows that the hormonal regulation of this fusion gene construct mimics that of the endogenous rat G6Pase gene in FAO cells (14) and primary hepatocytes (15) as well as a human G6Pase-CAT fusion gene expressed in H4IIE cells (25). Thus, dexametha-
sone stimulates mouse G6Pase-CAT expression, but this effect is blocked by insulin. This pattern of regulation is similar to that seen with a PEPCK-CAT reporter gene containing the promoter region from −486 to +69 (Fig. 1A; Ref. 24).

Insulin Represses Basal G6Pase-CAT but Not PEPCK-CAT Fusion Gene Expression—Insulin also inhibits the basal expression of the endogenous rat G6Pase and PEPCK genes in hepatoma cells (14, 26, 27). However, insulin represses only dexamethasone-stimulated, not basal PEPCK-CAT expression, following either transient (Fig. 1B) or stable (26) transfection. By contrast, insulin strongly represses both the dexametha-
sone-stimulated and the basal expression of a transiently transfected mouse G6Pase-CAT fusion gene (Fig. 1B). The rea-
son for this discrepancy with respect to PEPCK regulation is unknown. However, as a consequence of this loss of appropriate basal regulation of PEPCK-CAT fusion gene expression, the identification of an IRS in the PEPCK promoter has proved to be a difficult problem (1). This is because of the need to elevate basal PEPCK-CAT expression, using dexamethasone or cAMP, to be able to study the negative effect of insulin (26). The potential co-localization of positive cis-acting elements re-
quired for the effects of dexamethasone and cAMP with nega-
tive IRSs has made the interpretation of such experiments difficult (1).

Deletion of the G6Pase Promoter Sequence between −271 and −159 Abolishes the Inhibitory Effect of Insulin on G6Pase-CAT Fusion Gene Expression—We sought to make use of the observation that insulin inhibits basal G6Pase-CAT expression to define a region of the promoter required for this action of insulin. The ability of insulin to inhibit the basal expression of a series of 5′-truncated G6Pase-CAT fusion genes was analyzed by transient transfection of HepG2 cells (Fig. 5). Although the magnitude of the insulin effect is greater in H4IIE cells (Fig. 1), basal G6Pase-CAT expression is higher in HepG2 cells (data not shown). In these experiments, an expression vector for the insulin receptor was co-transfected with the G6Pase-CAT fu-
sion gene, since this has been shown to enhance the regulation of gene transcription by insulin in cases where the number of endogenous receptors is low (28). Fig. 2A shows that deletion of
the G6Pase promoter sequence between −271 and −199 (Region A) partially reduces the inhibitory effect of insulin, whereas deletion of additional sequences between −198 and −159 (Region B) completely abolishes the remaining effect of insulin on G6Pase-CAT fusion gene expression. These results are not explained by a loss of basal G6Pase-CAT expression (Fig. 2B). The simplest interpretations of these data are that either (i) Regions A and B each contain an independent IRS, or (ii) only Region B contains an IRS whereas Region A acts as an accessory element that enhances the action of a single IRS located in Region B. The latter is a common phenomenon in cAMP- and glucocorticoid-regulated gene transcription (29), but this arrangement would be entirely novel with respect to insulin action on gene transcription (1).

**The G6Pase Promoter Sequence between −197 and −159 Can Confer an Inhibitory Effect of Insulin on the Expression of a Heterologous Fusion Gene**—To determine whether Region B of the G6Pase promoter is sufficient to mediate an effect of insulin on transcription, double-stranded oligonucleotides representing this sequence (Fig. 3A) were synthesized and ligated into the BamHI site of the heterologous TKC-VI vector (19). This vector has previously been used to define an inhibitory sterol-responsive element in the low density lipoprotein receptor gene (20, 30–34). Like G6Pase, the molecule is glucose-6-phosphatase Insulin Response Sequence (GINS) and an inhibitory IRS in the PEPCK promoter responsive element in the low density lipoprotein receptor gene (30) and conserved between mouse and human. In addition, this region contains no homology with either Region B or any of the known IRSs in other genes (1). Additional experimentation will be required to determine whether Region A contains an independent IRS or simply enhances the action of the IRS in Region B.

The expression of insulin-regulated genes would be expected to change in patients with NIDDM as a consequence of insulin resistance (1, 5, 6). In fact, since insulin normally inhibits G6Pase and PEPCK gene transcription, the overexpression of these genes, as a consequence of insulin resistance, may in part explain the increased rate of gluconeogenesis, and HGP that is the major cause of fasting hyperglycemia in NIDDM (36). Indeed, the activity of the hepatic "glucose cycle," between G6Pase and glucokinase, is altered in both patients with NIDDM (37) and in animal models of diabetes (13), and overexpression of the PEPCK gene in HepG2 cells (38) and transgenic mice (39) does result in unrestrained HGP.

Although the evidence currently only supports a secondary role for altered insulin-regulated gene expression in NIDDM, the first example of a disease state resulting from a primary defect in insulin-regulated gene transcription has recently been elucidated. Thus, hepatic ApoCIII gene transcription is normally inhibited by insulin (40), but some patients with hypertriglyceridemia have a mutation in the ApoCIII IRS that abolishes the ability of insulin to suppress ApoCIII gene expression (Fig. 3B). These results suggest that the G6Pase promoter region between −197 and −159 contains an IRS and that insulin inhibits the transcription of the G6Pase gene, at least in part, through the same IRS and, presumably, the same trans-acting factor that mediates the effect of insulin on transcription of the PEPCK, TAT, IGFBP-1, and ApoCIII genes. Several proteins have been identified that bind this IRS, including members of the C/EBP (22) and HNF-3 (20, 31, 35) transcription factor families. However, in no case does the binding of one of these proteins correlate with the effect of insulin (20). Instead, it has been hypothesized that an unidentified factor mediates the negative effect of insulin through this element, perhaps by interfering with the binding of HNF-3 (1).

The mouse G6Pase promoter sequence between −197 and −159 (Region B; Fig. 2) is perfectly conserved in the rat G6Pase promoter (Fig. 3A; Ref. 15) and, with the exception of one base pair, also conserved in the human G6Pase promoter (Fig. 3A; Ref. 25). By contrast, the mouse G6Pase promoter sequence between −271 and −199 (Region A; Fig. 2) is only partially conserved between mouse and human. In addition, this region contains no homology with either Region B or any of the known IRSs in other genes (1). Additional experimentation will be required to determine whether Region A contains an independent IRS or simply enhances the action of the IRS in Region B.

**Glucose-6-phosphatase Insulin Response Sequence**

**Fig. 2.** Deletion of the G6Pase promoter sequence between −271 and −159 abolishes the inhibitory effect of insulin on G6Pase-CAT fusion gene expression. A, HepG2 cells were transiently co-transfected, as described previously (22), with G6Pase-CAT fusion genes (15 µg) containing various lengths of promoter sequence and an expression vector (5 µg) encoding the insulin receptor. Following transfection cells were incubated for 18–20 h in serum-free medium in the presence or absence of 100 nM insulin. The cells were then harvested and CAT activity asayed as described under "Experimental Procedures." Results are presented as the ratio of CAT activities in insulin-treated versus control cells (expressed as percent change) and represent the mean ± S.E. of four to nine experiments, in which each construct was assayed in duplicate. B, HepG2 cells were transiently transfected as described above except that an expression vector (2.5 µg) encoding β-galactosidase was also included. Following transfection cells were incubated for 18–20 h in serum-free medium. The cells were then harvested and CAT activity, β-galactosidase activity or the protein concentration in the cell lysate as shown. Results are expressed as a percentage relative to the −751 construct and represent the mean ± S.E. of three experiments, in which each construct was assayed in duplicate.
transcription (32). Experiments in transgenic mice demonstrated that the resulting overexpression of ApoCIII is sufficient to cause hypertriglyceridemia (41). We have previously postulated that a similar mutation in the PEPCK IRS could contribute to increased HGP (1, 6). However, a recent study failed to detect such mutations in a population of human subjects with NIDDM (42). Moreover, a defect in insulin-regulated PEPCK gene transcription, while it could explain the increase in HGP could not, by itself, explain hepatic insulin resistance. Nevertheless, changes in gene expression are likely to play a major role in the pathophysiology of NIDDM as suggested by the recent identification of mutations in the transcription factors HNF-1 and HNF-4, resulting in a form of NIDDM called maturity-onset diabetes of the young (43, 44).

In summary, this paper describes the identification of a multicomponent IRS in the mouse G6Pase promoter. Future studies will focus on the identification of the cis-acting elements that mediate the stimulatory effect of glucocorticoids and cAMP on G6Pase transcription with the ultimate aim of understanding how insulin signaling through the G6Pase IRS blocks this induction.

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REFERENCES

1. O’Brien, R. M., and Granner, D. K. (1996) Physiol. Rev. 76, 1109–1161
2. Rosseler, W. J., Vandenbark, G. R., and Hanson, R. W. (1988) J. Biol. Chem. 263, 9063–9066
3. Waterman, M. R. (1994) J. Biol. Chem. 269, 27763–27768
4. Rahmoe, D., and Herrlich, P. (1990) Pharmacol. Ther. 48, 157–188
5. DeFranzo, R. A., Bonadonna, R. C., and Ferrannini, E. (1992) Diabetes Care 15, 318–368
6. Granner, D. K., and O’Brien, R. M. (1992) Diabetes Care 15, 369–395
7. Burchell, A. (1990) FASEB J. 4, 2978–2988
8. Pilakis, S. J., and Granner, D. K. (1992) Annu. Rev. Physiol. 54, 885–909
9. Burchell, A., and Cain, D. I. (1985) Diabetesologia 28, 852–856
10. Maistrelli, D., Barralii, N., Chen, W., Hu, M., and Rossetti, L. (1996) J. Biol. Chem. 271, 9871–9874
11. Liu, Z., Barrett, E. J., Dalkin, A. C., Zwart, A. D., and Chou, J. Y. (1994) Glucose-6-phosphatase Insulin Response Sequence Glucagon-like peptide-1 (GLP-1) receptor.
12. Haber, B. A., Chiu, S., Chuang, E., Buiokuisen, W., Naji, A., and Taub, R. (1995) J. Clin. Invest. 95, 832–841
13. Yamamoto, and Jonathan Whittaker for generous gifts of the PEPCK-IRS promoter in either the correct (C) or mutated (MUT) G6Pase promoter sequence between 2A159 can confer an inhibitory effect of insulin on the expression of a heterologous fusion gene. A comparison of the mouse G6Pase promoter sequence between −197 and −159 with the equivalent sequence in the rat and human G6Pase genes. The single base pair change in the human sequence is indicated with a black dot. The homologies with the PEPCK/GFPB-1/TAT/ApoCIII IRSs are boxed. B, H4IIE cells were transiently transfected, as described previously (20), with either the basic TK-VI vector or constructs in which oligonucleotides representing the wild-type (WT) or mutated (MUT) mouse G6Pase promoter sequence from −197 to −159 shown in A had been ligated into the BamHI site of the TK promoter in either the correct (C) or inverted (I) orientation relative to that in the G6Pase promoter. Following transfection cells were incubated for 18–20 h in serum-free medium in the presence or absence of 10 nM insulin. The cells were then harvested and CAT activity assayed as described under “Experimental Procedures.” Results are presented as the ratio of CAT activities in insulin-treated versus control cells (expressed as percent control) and represent the mean of ± S.E. of four experiments, in which each construct was assayed in duplicate.

FIG. 3. The G6Pase promoter sequence between −197 and −159 can confer an inhibitory effect of insulin on the expression of a heterologous fusion gene. A, comparison of the mouse G6Pase promoter sequence between −197 and −159 with the equivalent sequence in the rat and human G6Pase genes. The single base pair change in the human sequence is indicated with a black dot. The homologies with the PEPCK/GFPB-1/TAT/ApoCIII IRSs are boxed. B, H4IIE cells were transiently transfected, as described previously (20), with either the basic TK-VI vector or constructs in which oligonucleotides representing the wild-type (WT) or mutated (MUT) mouse G6Pase promoter sequence from −197 to −159 shown in A had been ligated into the BamHI site of the TK promoter in either the correct (C) or inverted (I) orientation relative to that in the G6Pase promoter. Following transfection cells were incubated for 18–20 h in serum-free medium in the presence or absence of 10 nM insulin. The cells were then harvested and CAT activity assayed as described under “Experimental Procedures.” Results are presented as the ratio of CAT activities in insulin-treated versus control cells (expressed as percent control) and represent the mean of ± S.E. of four experiments, in which each construct was assayed in duplicate.

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