The Molecular Physiology of Hepatic Nuclear Factor 3 in the Regulation of Gluconeogenesis*

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Glucocorticoids stimulate gluconeogenesis by increasing the rate of transcription of genes that encode gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase. Previous studies have shown that hepatic nuclear factor 3 (HNF3) is required as an accessory factor for several glucocorticoid-stimulated genes, including PEPCK. Here, we show that adenovirus-mediated expression of an HNF3β protein with a deleted C-terminal transactivation domain (HNF3βΔC) reduces the glucocorticoid-induced expression of the PEPCK and glucose-6-phosphatase genes in H4IIE hepatoma cells. Furthermore, expression of this truncated HNF3 protein results in a proportionate reduction of glucocorticoid-stimulated glucose production from lactate and pyruvate in these cells. The expression of HNF3βΔN, in which the N-terminal transactivation domain is deleted, does not exhibit any of these effects. These results provide direct evidence that members of the HNF3 family are required for proper regulation of hepatic gluconeogenesis. Modulation of the function of the HNF3 family of proteins might be used to reduce the excessive hepatic production of glucose that is an important pathophysiologic feature of diabetes mellitus.

The stimulation of gluconeogenesis is one of the important functions of glucocorticoids. This effect is due in part to the ability of glucocorticoids to induce the transcription of genes that encode gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) (1, 2). The mechanism by which glucocorticoids regulate transcription of the PEPCK gene has been studied in detail. A glucocorticoid response unit (GRU) that consists of two glucocorticoid receptor (GR)-binding sites (GR1 and GR2) and four accessory elements (gAF1, gAF2, gAF3, and cyclic AMP response element) in the PEPCK gene promoter is required for a complete glucocorticoid response (3–7). A mutation of any of these accessory elements results in a 50% reduction of glucocorticoid-stimulated PEPCK gene transcription, and a combination of any two mutations of the accessory elements abolishes the glucocorticoid response (3, 5, 8). It follows from these results that GR1 and GR2 cannot mediate the glucocorticoid response by themselves in the context of the PEPCK gene promoter. Indeed, GR1 and GR2, either alone or in combination, cannot mediate a glucocorticoid response when placed in the context of a heterologous promoter (9). Thus, the glucocorticoid response of the PEPCK gene is completely dependent on accessory factor elements and the proteins that bind to these sites. In recent studies we have shown that chicken ovalbumin upstream promoter transcription factor and HNF4 bind to gAF1 (4), HNF3 binds to gAF2 (7), chicken ovalbumin upstream promoter transcription factor binds to gAF3 (5), and CAAT enhancer-binding protein binds to the cyclic AMP response element (10).

Members of the hepatic nuclear factor 3 (HNF3) family, HNF3α, β, and γ, play a central role in the regulation of a number of genes that are involved in various aspects of cellular metabolism (7, 11–14). Much of this regulation is accomplished through N- and C-terminal transactivation domains (15). In addition, the C-terminal domain is required for accessory factor activity in the PEPCK gene GRU, whereas the N-terminal transactivation domain does not support this function (16). Interestingly, HNF3 also functions as an accessory factor in many other GRUs, such as those located in the tyrosine aminotransferase, insulin-like growth factor-binding protein-1, and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene promoters (12–14). Thus, the functional interaction between HNF3 and GR appears to be a common mechanism for glucocorticoid-regulated gene transcription. Although a considerable amount is known about the organization of the GRUs of various promoters, little is known about how the different components actually influence hepatic glucose production from gluconeogenesis.

In this study recombinant adenoviruses that express structural variants of HNF3β were constructed to study the role this family of proteins plays in the regulation of gluconeogenesis in H4IIE hepatoma cells. We show that adenovirus-mediated ex-

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pression of a form of HNF3β that lacks the C-terminal transactivation domain results in a decrease of glucocorticoid-stimulated PEPCK and G-6-Pase gene transcription. Exposure of H4IIE hepatoma cells to the same adenovirus reduces glucose production in proportion to the decreased rate of expression of these genes. By contrast, an HNF3β variant protein that lacks the N-terminal transactivation domain does not support the glucocorticoid response on gene expression, nor does it influence gluconeogenesis. These results confirm the importance of HNF3β in the regulation of glucocorticoid-stimulated gene expression and provide direct evidence for the critical role of the protein in the regulation of gluconeogenesis.

MATERIALS AND METHODS

Cell Culture, Treatment with Recombinant Adenovirus, and CAT Assays—The maintenance of H4IIE and H11C cells and the measurement of CAT activity have been previously described (3, 17). Cells were treated with an appropriate volume of cell lysates containing recombinant adenovirus in fresh Dulbecco’s modified essential medium supplemented with 2.5% newborn calf serum and 2.5% fetal bovine serum for 16–24 h (see below). After washing with phosphate-buffered saline, cells were incubated with serum free Dulbecco’s modified essential medium with or without 500 nM dexamethasone (DEX) for another 16–24 h.

Preparation of Recombinant Adenovirus—A cDNA that encodes amino acids 1–366 of HNF3β (HNF3βAC) and another that encodes amino acids 52–458 of HNF3β (HNF3βNC) were cloned into the pJM17 plasmid to construct recombinant adenoviruses by homologous recombination as described previously (18). The adenoviruses were named Ad-HNF3βAC and Ad-HNF3βNC, respectively. The construction of a recombinant adenovirus containing the cDNA that encodes the Escherichia coli β-galactosidase gene (Ad-β-Gal) was described previously (19). The amount of Ad-β-Gal virus used was determined by first finding the volume of clarified 293 cell lysate that stained more than 80% of a monolayer of HeLa cells after fixation and treatment with 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-gal) (20). The amount of Ad-HNF3βAC and Ad-HNF3βNC viruses used was determined from a dose-response experiment in which the maximum amount of virus that did not demonstrate toxicity to the cells was established. For the Ad-HNF3βAC virus, this volume of clarified lysate inhibited dexamethasone-stimulated PEPCK gene expression from semiconfluent HL1C cells in a 100-cm2 dish by 50%, as measured by CAT activity. Overexpression of protein from both viruses was confirmed by gel shift analysis. Expression levels of HNF3βAC and HNF3βNC from the selected amount of virus were approximately equal as determined by this method, and both proteins were expressed in excess of endogenous HNF3β.

Gel Mobility Shift Assays—The preparation of whole cell lysates and the method of performing the gel mobility shift assays have been previously described (16). The double-stranded oligonucleotide used in the gel mobility shift assays has the sequence 5′-CAGTACCAAACCAACATTATTTTTGAAACAC-3′ (13). The polyclonal HNF3β antibody was obtained from James Darnell of Rockefeller University.

Ribonuclease Protection Assay—Total cellular RNA was isolated from H4IIE cells using TRI REAGENT (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. RNase protection assays were performed according to the instructions provided with the Ambion (Austin, TX) RPAI kit. The rat PEPC and G-6-Pase RNA probes were generated from polymerase chain reactions in which the downstream primer contained the T7 promoter. A 5′-paliquot of the polymerase chain reaction was added directly to the components of the Ambion Maxiscript kit with [α-32P]UTP, to produce radiolabeled RNA. The rat β-actin control probe purchased from Ambion generated a 126-base pair fragment after digestion with RNase A and T1. The sequences of the oligonucleotides used for the probes for rat PEPC (150 base pairs after digestion) and rat G-6-Pase (175 base pairs after digestion) are 5′-GGTGTTGGAAATGTTTAGGTG (upstream) and 5′-GGATCCTTAATACGACTTCACTATAGGGAGGACCGTCCACTACACGGG (downstream) for PEPC and 5′-CAGTGCTAGCCTGCTACTCT (downstream) and 5′-GGATCCTTAATACGACTTCACTATAGGGAGGACCGTCCACTACACGGG (downstream) for G-6-Pase.

Glucose Production Assay—H4IIE cells were incubated in 100-cm2 plates 3 h at 37 °C in 15% atmospheric air/5% CO2 in 5 ml of glucose production buffer (glucose-free Dulbecco’s modified essential medium, pH 7.4, containing 20 mM sodium lactate and 2 mM sodium pyruvate without phenol red). At the end of this incubation, 0.5 ml of medium was taken to measure the glucose concentration in the culture medium using a glucose assay kit (Sigma 510-A). A 2-fold concentration of the kit reagents was used to increase the sensitivity. Cells were collected and lysed, and the total protein concentration was measured (Bio-Rad) to correct for cell count. Statistical evaluations were obtained using the Student’s t test method.

RESULTS AND DISCUSSION

Previous experiments have shown that HNF3β, by binding to the gAF2 element in the GRU, serves as an accessory factor for glucocorticoid-mediated activation of PEPC and gene expression. This effect requires the C-terminal transactivation domain of HNF3β but is not dependent on the N-terminal transactivation domain (16). We reasoned that a protein lacking the C-terminal transactivation domain would bind DNA and compete with the endogenous protein with regard to glucocorticoid accessory factor activity. This protein, if abundantly expressed in H4IIE cells, should blunt the response of the PEPC gene to glucocorticoids in a manner equivalent to a mutation or deletion of gAF2 (3, 16, 21). If this gene is centrally involved in gluconeogenesis, as is suggested by metabolic control strength studies, glucocorticoid-mediated glucose production from noncarbohydrate precursors should also be decreased (22). Accordingly, a recombinant adenovirus (Ad-HNF3βAC) that lacks this C-terminal domain was constructed. A second recombinant adenovirus was prepared in which the N-terminal transactivation domain of HNF3β was deleted (Ad-HNF3βAN). The latter construct served as a control because it retains accessory factor activity (16). A gel mobility shift assay was used to confirm that these two HNF3β mutants/proteins are expressed in H4IIE hepatoma cells after the cells are exposed to the adenoviruses. The C-terminal truncated protein is smaller than the N-terminal truncated protein, thus the protein-DNA complex formed from extracts of HNF3β-A-treated cells migrates faster than its counterpart from HNF3βAN-treated cells (Fig. 1, compare lanes 7 and 10). The formation of both protein-DNA complexes was blocked by adding an antisense directed against HNF3β but not by an antisense directed against LexA (Fig. 1, lanes 7–9 and 10–12). These experiments confirm that the truncated HNF3β proteins are expressed in H4IIE cells. Furthermore, the expression level of these two HNF3β proteins was approximately equal, and both were expressed in excess of the endog-
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significant difference (p

adenous HNF3β (Fig. 1, lanes 1–6). The treatment of H4IIE cells with a recombinant adenoavirus that contains a cDNA that expresses β-galactosidase (Ad-β-Gal) did not influence the binding pattern of HNF3β proteins in the gel mobility shift assay (Fig. 1, compare lanes 1–3 with lanes 4–6).

The HL1C cell line is a stable transfectant of H4IIE cells that contains the PEPCK gene promoter from −2100 to +69, relative to the transcription start site, ligated to the CAT reporter gene. The regulation of CAT gene expression and activity recapitulates the hormonal regulation of the endogenous PEPCK gene in these cells so they are very useful in studying PEPCK glucocorticoid response and that the N-terminal domain is not necessary (16). By contrast, the DEX response of HL1C cells was unaffected (Fig. 2). As previously demonstrated, treatment of HL1C cells with the Ad-β-Gal adenoavirus does not influence the DEX response of these cells (Fig. 2 and Ref. 20). The DEX response was, however, reduced by about 50% when HL1C cells were treated with Ad-HNF3ΔC. By contrast, the DEX response of HL1C cells treated with HNF3ΔN was unaffected (Fig. 2). These results are consistent with our previous findings showing that a deletion or a mutation of the HNF3-binding site (gAF2) of the PEPCK gene promoter resulted in a 50–70% reduction of the response of this gene to glucocorticoids (3, 7, 8). These results also confirm the observation that the C-terminal transactivation domain is required for the accessory activity of the PEPCK glucocorticoid response and that the N-terminal domain is not necessary (16).

H4IIE cells were treated with Ad-β-Gal, Ad-HNF3ΔC, or Ad-HNF3ΔN to determine the role HNF3β plays in the regulation of endogenous PEPCK gene expression by glucocorticoids. Ribonuclease protection assays were used to measure PEPCK mRNA (Fig. 3). Overnight exposure of H4IIE cells to DEX resulted in a 3-fold induction of PEPCK mRNA (Fig. 3, compare lanes 1 and 2). The prior treatment of the cells with either Ad-β-Gal (lanes 3 and 4) or Ad-HNF3ΔN (lanes 7 and 8) had no significant effect on this induction, which is due to an increased rate of transcription of the gene (24). By contrast, treatment of cells with Ad-HNF3ΔC decreased the glucocorticoid-stimulated PEPCK mRNA response by about 50% and the basal expression of the PEPCK gene by about 20% (lanes 5 and 6). β-Actin mRNA was not affected by DEX or by the prior treatment with any of the adenovirus constructs. These results demonstrate the importance of HNF3 in the regulation of the endogenous PEPCK gene by glucocorticoids and are consistent with the conclusions derived from previous studies (7, 16). In addition, expression of the PEPCK gene is markedly reduced when the HNF3 DNA-binding domain, which does not contain either of the transactivation domains, is stably transfected into hepatoma cells (25). Furthermore, expression of the PEPCK gene is reduced in mice carrying a null mutation of the gene encoding HNF3γ (26).

The rate of transcription of the G-6-Pase gene, which encodes another rate-controlling gluconeogenic enzyme, is also stimulated by glucocorticoids (27). There are several HNF3-binding sites in the G-6-Pase gene promoter (28), so we tested for effects of the HNF3β mutants on the expression of the endogenous G-6-Pase gene (Fig. 3). Adenovirus-mediated expression of HNF3ΔC, but not HNF3ΔN, repressed glucocorticoid-stimulated G-6-Pase mRNA levels (Fig. 3, compare lanes 5 and 6 with lanes 7 and 8). Although HNF3 is not known to be an accessory factor for the glucocorticoid response of the G-6-Pase gene, the results here suggest that it may serve this function. This would not be surprising because HNF3 is an accessory factor for the glucocorticoid response of the tyrosine aminotransferase, insulin-like growth factor-binding protein-1, and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase genes (12–14).

Because HNF3ΔC reduced the glucocorticoid-mediated induction of PEPCK and G-6-Pase gene expression, we predicted that this treatment would also decrease glucocorticoid-stimulated gluconeogenesis. We therefore measured glucose production in H4IIE cells after exposure to the recombinant adeno-
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The reduction of glucagon gene expression in pancreatic cells (100%). Data represent the means (as described under “Materials and Methods.” Results are presented as the glucose concentration was measured in the extracellular medium determined as described under “Materials and Methods.” The cells were incubated in glucose-free Dulbecco’s modified Eagle’s medium, pH 7.4, supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate for 3 h. The glucose concentration was measured in the extracellular medium as described under “Materials and Methods.” Results are presented as percentages relative to the glucose produced by DEX-treated H4IIE cells (100%). Data represent the means (± S.E.) glucose, corrected for total cell protein, of at least three experiments. The DEX response of cells transfected with Ad-HNF3βAC was significantly different (p < 0.0001) from that of cells transfected with Ad-β-Gal (double asterisks). The DEX response of cells transfected with Ad-HNF3βAN was different (p < 0.05) from cells transfected with Ad-β-Gal.

ruses. The low basal glucose production from gluconeogenesis in H4IIE cells is increased 5-fold by an overnight exposure of the cells to DEX (Fig. 4). Adenovirus-mediated expression of HNF3βAC reduced glucocorticoid-induced glucose production in a dose-dependent manner (Fig. 4). By contrast, the expression of HNF3βAN had a small stimulatory effect on glucocorticoid response, may provide an opportunity for the pharmacological manipulation of gluconeogenesis.

In summary, this report provides direct evidence for the role of the HNF3 family of proteins in the regulation of gluconeogenesis. The importance of HNF3 in glucocorticoid-stimulated expression of the PEPCK and G-6-Pase genes suggests that modulation of the function of HNF3 could influence the excessive rate of hepatic gluconeogenesis that is part of the pathophysiology of type 2 diabetes mellitus. As an extension of this view, the intricate structure of the PEPCK GRU, which requires many transcription factors other than GR for a complete glucocorticoid response, may provide an opportunity for the pharmacological manipulation of gluconeogenesis.

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