Cloning of the Rat Pyruvate Dehydrogenase Kinase 4 Gene Promoter

ACTIVATION OF PYRUVATE DEHYDROGENASE KINASE 4 BY THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ COACTIVATOR*

Received for publication, February 28, 2005, and in revised form, June 1, 2005
Published, JBC Papers in Press, June 20, 2005, DOI 10.1074/jbc.M502236200

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The pyruvate dehydrogenase complex catalyzes the conversion of pyruvate to acetyl-CoA in mitochondria and is a key regulatory enzyme in the metabolism of glucose to acetyl-CoA. Phosphorylation of pyruvate dehydrogenase by the pyruvate dehydrogenase kinases (PDK) inhibits pyruvate dehydrogenase complex activity. There are four PDK isoforms, and expression of PDK4 and PDK2 genes is elevated in starvation and diabetes, allowing glucose to be conserved while fatty acid oxidation is increased. In these studies we have investigated the transcriptional mechanisms by which the expression of the PDK4 gene is increased. The peroxisome proliferator-activated receptor γ coactivator (PGC-1α) stimulates the expression of genes involved in hepatic gluconeogenesis and mitochondrial fatty acid oxidation. We have found that PGC-1α will induce the expression of both the PDK2 and PDK4 genes in primary rat hepatocytes and ventricular myocytes. We cloned the promoter for the rat PDK4 gene. Hepatic nuclear factor 4 (HNF4), which activates many genes in the liver, will induce PDK4 expression. Although HNF4 and PGC-1α interact to stimulate several genes encoding gluconeogenic enzymes, the induction of PDK4 does not involve interactions of PGC-1α with HNF4. Using the chromatin immunoprecipitation assay, we have demonstrated that HNF4 and PGC-1α are associated with the PDK4 gene in vivo. Our data suggest that by inducing PDK genes PGC-1α will direct pyruvate away from metabolism into acetyl-CoA and toward the formation of oxaloacetate and into the gluconeogenic pathway.

The pyruvate dehydrogenase complex (PDC) catalyzes the formation of acetyl-CoA from pyruvate. PDC is an important regulatory enzyme in the metabolism of glucose to acetyl-CoA (1). The activity of PDC is controlled in part through its phosphorylation and dephosphorylation by pyruvate dehydrogenase kinases (PDKs), and pyruvate dehydrogenase phosphatases, respectively (2, 3). There are three serine phosphorylation sites on the α subunit of pyruvate dehydrogenase (E1) that are targeted by PDKs. Phosphorylation will completely inhibit PDC activity (1, 2). The PDKs are subject to short term regulation including inhibition by the PDC substrates pyruvate and NAD+ as well as stimulation by the PDC products NADH and acetyl-CoA. The acetyl-CoA, which stimulates PDK4 activity, may be derived from fatty acid or glucose oxidation (2). In the liver PDC activity is high after a high carbohydrate diet, and the acetyl-CoA generated from PDC is utilized for the synthesis of long chain fatty acids (4).

There are four PDK isoforms, and two of these, PDK2 and PDK4, are highly expressed in the liver, heart, and kidney (4–8). These PDK isoforms are regulated in the long term by changes in gene expression. Expression of both PDK2 and PDK4 genes are induced in starvation and by streptozotocin-induced diabetes (2, 4). Several factors including long chain fatty acids, glucocorticoids, and peroxisome proliferator-activator receptor agonists will increase transcription of the PDK4 gene (5, 7). The expression of both PDK2 and PDK4 is inhibited by insulin, although the PDK2 gene is more sensitive than the PDK4 gene to insulin inhibition in Morris hepatoma 7800 C1 cells (7).

Peroxisome proliferator-activated receptor γ coactivator (PGC-1α) was first cloned from brown adipose tissue as a transcriptional coactivator for peroxisomal proliferator-activated receptor γ (9). PGC-1α is highly expressed in tissues with high metabolic rates including heart, muscle, and brown adipose tissue (10). Overexpression of PGC-1α in heart and muscle promotes mitochondrial biogenesis and elevated fatty acid oxidation (11). The heart genes encoding the fatty acid oxidation enzymes, such as medium chain acyl-CoA dehydrogenase and the “muscle” isomerase of carnitine palmitoyltransferase-I (CPT-Iβ), are stimulated by PGC-1α (12). In cardiac myocytes PGC-1α is increased at birth when there is a switch from glucose to fatty acid utilization, and PGC-1α is elevated in the heart of fasted animals (12).

In the liver transcription of PGC-1α is induced by dexamethasone, cAMP, and thyroid hormone (13, 14). PGC-1α can induce the “liver isoform” of the CPT-I (CPT-Iα) gene (13). Although PGC-1α is expressed at low levels in the liver of fed animals, after an overnight fast PGC-1α abundance is elevated (13, 15). PGC-1α is increased in situations where hepatic mitochondrial fatty acid oxidation and CPT-Iα activity are stimulated such as fasting, streptozotocin-induced diabetes, and hyperthyroidism (13, 14, 16). Overexpression of PGC-1α will

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* The abbreviations used are: PDC, pyruvate dehydrogenase complex; PGC-1, peroxisome proliferator-activated receptor γ coactivator; PDK, pyruvate dehydrogenase kinase; CPT, carnitine palmitoyltransferase; SCD, stearoyl-CoA desaturase; PEPCK, phosphoenolpyruvate carboxykinase; HNF-4, hepatic nuclear factor 4; FOXO1, forkhead transcriptional factor; ChIP, chromatin immunoprecipitation assay; GFP, gene-specific primer; PDK4-luc, PDK4-luciferase; RACE, rapid amplification of cDNA ends; r, rat; GFP, green fluorescent protein.
induce the expression of the phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase genes, resulting in accelerated hepatic gluconeogenesis (17, 18). PGC-1α mediates many of its actions on the gluconeogenic pathway through interactions with nuclear receptors such as hepatic nuclear factor-4 (HNF4), the glucocorticoid receptor, the forkhead transcription factor (FKHR/FOXO1), and others (10, 13, 15, 17). The interactions with HNF4 and the glucocorticoid receptor are mediated in part through the AF2 domain in the nuclear receptor.

We hypothesized that PGC-1α might further regulate pyruvate metabolism by inducing the PDK genes. Here, we show that PGC-1α can induce PDK2 and PDK4 gene expression in primary rat hepatocytes and myocytes. We cloned the rat PDK4 promoter and examined the regulation of the PDK4 gene by PGC-1α.

**MATERIALS AND METHODS**

**Cloning of the Rat PDK4 Promoter.—** Genomic DNA was prepared from rat tails using a Qiagen genomic DNA isolation kit (QiAamp DNA kit 51904). A forward primer containing the sequence 5′-gttgcagcactctcagcagtctcagcag (+2989/+2965) and a reverse primer, 5′-ggggagggaggtggac-agggagggagagggagggagggagggagggagggagggaga (2767/2742), were used in polymerase chain reactions to amplify 2789 nucleotides of the promoter and 87 nucleotides of the first exon from the rat PDK4 gene.

**Identification of the Transcriptional Start Site by Rapid Amplification of cDNA Ends (RACE) —** Transcription start site determinations were performed on RNase I-treated total RNA isolated from rat hepatocytes (19). RNA was isolated with RNA-Stat 60 reagent (Tel-Test) as we have described previously (19). The cDNA was prepared using the 5′ RACE system for rapid amplification of cDNA ends (Invitrogen #18374-058). The following rat PDK4 gene-specific primers were designed with Primer3_www.cgi v 0.2 software. The gene-specific primer 1 (GSP1) 5′-gctcctaacattcttgctctg-3′ was used for reverse transcription to create a fragment of approximately 1700 bp. The GSP2, 5′-gttgcagcactcctctttagttttctcagcag (+2989/+2965), was used in the nested PCR reactions to identify the 5′ end of the mRNA. PCR products were cloned into the pCR4-TOPO vector from the TOPO TA cloning vector system (Invitrogen). PCR products were sequenced at the University of Tennessee Molecular Resource Center.

**Transient Transfection of Luciferase Vectors—** PDK4-luciferase (PDK4-luc) constructs were transiently transfected into HepG2 cells by the calcium phosphate method (20). Transfections included 2 μg of PDK4-luciferase along with TK-Rev and mammalian expression vectors for either HNF4 or PGC-1α. Cells were transfected in Dulbecco’s modified Eagle’s medium containing 5% calf serum, 5% fetal calf serum, modified Eagle’s medium containing 5% calf serum, 5% fetal calf serum (21). The PCR products were sequenced using the pCR4-TOPO vector from the TOPO TA cloning vector system (Invitrogen #18374-058). The forward primer contains the sequence 5′-gctcctaacattcttgctctg. The altered nucleotides are underlined. All deletions and site-directed mutants were confirmed by DNA sequencing at the University of Tennessee Molecular Resource Center.

**Adenoviral Infection of Primary Rat Hepatocytes and Neonatal Cardiac Ventricular Myocytes—** Hepatocytes were obtained from livers of male Sprague-Dawley rats (Harlan) as we have reported previously (21). The cardiac myocytes were prepared from 2- and 3-day-old rats (22). The adenoviruses expressing green fluorescent protein (GFP) and PGC-1α were purified by cesium chloride purification (13, 23). Rat primary hepatocytes were plated in RPMI 1640 media containing 20 mM glucose in the absence of serum before infection. Ventricular myocytes were plated in Dulbecco’s modified Eagle’s medium containing 20 mM glucose. The hepatocytes and myocytes were infected at a multiplicity of infection of 50 (23). Sixteen hours after the infection the cells were changed to fresh medium. After infection for 48 h, RNA was isolated from the hepatocytes and myocytes.

**Western Analysis—** Rats were made diabetic by a single intraperitoneal injection of streptozotocin at 150 mg/kg of body weight (24). After 6 days of diabetes, the liver was harvested in PD buffer (40 mM Tris-Cl, pH 8.0, 50 mM NaCl, 0.5% Nonidet P-40, 6 mM EDTA, pH 8.0, 6 mM EGTA, pH 8.0, 1 mM dithiothreitol, and dithiol protease inhibitor mixture Sigma P8340). The liver samples were sonicated at 4°C for 10 min in a Microson sonicator (Misonix ZX2000). Cell membranes were removed by centrifugation for 25 min at 4°C. An equal amount of protein was loaded onto a 12% SDS-PAGE gel and transferred to a 0.45-μm pure nitrocellulose membrane (Bio-Rad). Blots were immunoblotted with primary antibodies (anti-PGC-1α, Santa Cruz sc-13067) in phosphate-buffered saline containing 5% nonfat dry milk powder and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Bio-Rad, 170-6651). Immunoreactive proteins were identified using Super Signal West Femto Chemiluminescence Substrate (Pierce).

**Real-time PCR—** cDNA was extracted from primary rat hepatocytes or cardiac myocytes using RNA-Stat-60 (Tel-Test) as described previously (14). The RNA was initially treated with DNase I (Ambion) at 37 °C for 1 h. The cDNA was subsequently treated with Superscript II reverse transcriptase. The tubes were incubated at 25 °C for 10 min, 37 °C for 1 h, and 70 °C for 15 min. The cDNA was stored at −20 °C until it was used to prepare real-time PCR reaction mixtures.

**TABLE I**

| Gene         | Primer                        | Sequence (5′→3′)                        |
|--------------|-------------------------------|----------------------------------------|
| PDK4 FP      | GATTACTAATGACCCCTCTTTCAAGTT  |
| PDK4 RP      | GACCTGCTCGTAAGATGTCACAATC    |
| PDK2 FP      | ACCCGGTTCTCCACCCGGAACAG      |
| PDK2 RP      | TAACTGCGGCTGATTGAAGAATTG    |
| CPT-I FP     | TCTCAGCAACATTGCATGCATTG     |
| CPT-I RP     | TACAACTCAGTCGGCAAGCTTT      |
| CPT-III RP   | GCCTCCGCAAGCTGACAGAG       |
| CPT-III FP   | CGTACATGGGGACTACATGACCC      |
| mHMG-CoA synthase | AGCAAAATGGCCCAACAATC   |
| mHMG-CoA synthase | AAACAGTGGGATCCTCTCTCTCT      |
| SCD-1 FP     | CCACCTGTCATCTCCTCTCTCTAAT   |
| SCD-1 RP     | AGGCCCTAAAGATTTGTCAAA       |
| SCD-2 FP     | CAGGTCGTCATCTCCTCTCTCTAAT   |
| SCD-2 RP     | TTTTCGTCACTATTTGTTCACA     |
| 18 S RNA + 452 FP | CTGCTCCACACACAGGAAGGA     |
| 18 S RNA + 524 FP | TTTTTCACTGACACCTCCCG      |
lated from rat liver nuclei in binding buffer containing 50 mM KCl, 25 mM Tris HCl, pH 7.4, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol (20, 23). The nonspecific competitor added to each binding reaction was 1 μg of a 1:1 ratio of poly(dI:dC)poly(dA:dT). Antibodies were added to the binding reactions before the addition of nuclear protein extract. The antibody to HNF4 (Santa Cruz, sc-8987) was purchased from Santa Cruz. Binding reaction before the addition of nuclear protein extract. The antibody to rat liver nuclei were isolated by the method of Gorski (25).

RESULTS

PGC-1α and PDK4 gene expression are elevated in the liver of diabetic and fasted animals (4, 13). Our first experiments were designed to determine whether PGC-1α could stimulate the expression of hepatic genes involved in glucose and lipid metabolism. We infected primary rat hepatocytes with adenoviruses expressing either GFP (Ad-GFP) or PGC-1α (Ad-PGC-1α). After 48 h, RNA was harvested, and the PDK2 and PDK4 mRNA abundance were measured by real-time PCR. As is shown in Fig. 1A, overexpression of PGC-1α increased the PDK4 mRNA abundance 6.2 ± 0.9-fold and the PDK2 mRNA abundance 9.0 ± 1.2-fold. PGC-1α stimulated the abundance of CPT-1α mRNA 4-fold. Interestingly, the expression of mitochondrial hydroxymethylglutaryl-CoA synthase, which is involved in ketogenesis, was not increased by PGC-1α overexpression (Fig. 1A). The expression of steroyl-CoA desaturases 1 and 2 (SCD-1 and SCD-2) were not increased by PGC-1α. Long chain fatty acids will increase transription of PDK4 (7). We tested whether the addition of long chain fatty acids would alter the ability of PGC-1α to stimulate the PDK4 or PDK2 genes. Hepatocytes were incubated with RPMI 1640 containing 20 mM glucose and either bovine serum albumin (BSA) or BSA with 350 μM oleate. Incubation with oleate increased basal expression of the PDK4 gene 2.5-fold in the cells infected with GFP (Fig. 1C). PGC-1α stimulated PDK4 gene expression an additional 2.5-fold in hepatocytes incubated with fatty acids as opposed to 4-fold with the bovine serum albumin alone (Fig. 1C). These experiments indicate that PGC-1α can induce PDK4 even when the PDK4 gene expression is elevated by fatty acids. On the other hand, the induction of PDK2 by PGC-1α was equal in the presence or absence of the fatty acids (data not shown).

Because PDK2 and PDK4 are elevated in the hearts of fasted animals, we tested the ability of PGC-1α to induce the expression of PDK4 and PDK2 mRNA in primary cardiac myocytes. Ventricular myocytes were prepared from newborn rats and infected with either Ad-GFP or Ad-PGC-1α. After infection, the myocytes were maintained in serum-free media containing 10 μM oleate. Incubation with 10 μM oleate increased basal expression of the PDK4 gene 1.7-fold. PGC-1α overexpression, whereas PDK2 was stimulated 3.5 ± 0.6-fold (Fig. 1B). The CPT-1α and CPT-1β isozymes were induced 4.5- and 3.3-fold respectively, whereas the mitochondrial hydroxymethylglutaryl-CoA synthase was not increased. Expression of SCD-1 but not SCD-2 was elevated by PGC-1α overexpression, whereas PDK2 was stimulated 3.5 ± 0.6-fold (Fig. 1B). The CPT-1α and CPT-1β isozymes were induced 4.5- and 3.3-fold respectively, whereas the mitochondrial hydroxymethylglutaryl-CoA synthase was not increased. Expression of SCD-1 but not SCD-2 was elevated by PGC-1α overexpression (Fig. 1B). We observed that the induction of PDK4 mRNA abundance by PGC-1α varied in ventricular myocytes under different culture conditions. When the myocytes were cultured in high glucose (20 mM) containing 5% calf serum, the PDK4 and PDK2 mRNA abundance was increased 31.7 ± 0.7- and 7.6 ± 2.4-fold, respectively (data not shown). However, altering the culture media did not affect the ability of PGC-1α to induce either the CPT-1α or CPT-1β isozymes, suggesting that this effect was limited to the PDK genes (data not shown). When the myocytes were cultured in media containing 5.5 mM glucose in the absence of serum, the PDK4 and PDK2 mRNA abundance was increased 7.5- and 4.5-fold, respectively, by PGC-1α. Overall, our data demonstrated that the PDK4 gene is highly regulated by PGC-1α. Given this extensive regulation of PDK4 and PDK2 mRNA abundance by PGC-1α, we cloned the promoter for the rat PDK4 gene. The rat PDK4 promoter was cloned using PCR amplification of rat genomic DNA with PDK4-specific primers. The PDK4 promoter

| Regions of PDK4 gene amplified | Forward primer (FP) | Reverse primer (RP) |
|--------------------------------|---------------------|--------------------|
| RatPDK4, ~1504/~1197          | aagttcttacaaggttg   | taagagataaactagt   |
| RatPDK4, ~578/~735           | caagatgaataagctat   | actaagagccatcagtc  |

**Table II**

The following primers were used for the ChIP assay to demonstrate interactions of PGC-1α and HNF-4 with the PDK4 gene. The following primers were used for the ChIP assay to demonstrate interactions of PGC-1α and HNF-4 with the PDK4 gene.
contained 2989 base pairs of 5'-flanking sequence. We confirmed the identity of the rat PDK4 promoter by RACE analysis using nested primers from the second exon of the PDK4 gene, which is within the protein-coding sequence (Fig. 2A). All the RACE products contained a single first exon, and we did not identify any alternate exons with this approach. Approximately 80% of the PCR reactions stopped at the adenosine nucleotides in the AGACA sequence at the end of the PDK4 mRNA abundance of the PGC-1α-infected cells relative to the GFP-infected cells. B, primary neonatal myocytes were infected with adenoviral vectors exactly as with the hepatocytes. Determination of RNA abundance from the myocytes was conducted as described under “Materials and Methods.” The data represent the average of three independent infections of myocytes. C, hepatocytes were incubated in RPMI 1640-containing bovine serum albumin (BSA) or BSA and 350 μM oleate. Infections with Ad-GFP and Ad-PGC-1α were conducted as described in A. RNA was harvested, and the PDK4 mRNA was assessed by real-time PCR. The data are the average of three independent infections of hepatocytes. D, hepatocytes were infected with Ad-PGC-1α at three different multiplicities of infection (MOI) as indicated above the Western blot. Proteins were prepared from whole liver extract of streptozotocin-induced diabetic and control rats. The PGC-1α abundance was determined by Western analysis, and the protein source is indicated above the immunoblot.

**FIG. 1.** PGC-1α induces PDK4 and PDK2 gene expression. A, primary rat hepatocytes were infected with adenoviral vectors expressing PGC-1α (Ad-PGC-1α). The infection conditions are described under “Materials and Methods.” After 48 h RNA was isolated, and the mRNA abundance of the various genes was measured by real-time PCR as described under “Materials and Methods.” 18 S rRNA was used as the control. The infections were repeated four times on independent preparations of hepatocytes. The data are expressed as-fold induction of mRNA abundance of the PGC-1α-infected cells relative to the GFP-infected cells. B, primary neonatal myocytes were infected with adenoviral vectors exactly as with the hepatocytes. Determination of RNA abundance from the myocytes was conducted as described under “Materials and Methods.” The data represent the average of three independent infections of myocytes. C, hepatocytes were incubated in RPMI 1640-containing bovine serum albumin (BSA) or BSA and 350 μM oleate. Infections with Ad-GFP and Ad-PGC-1α were conducted as described in A. RNA was harvested, and the PDK4 mRNA was assessed by real-time PCR. The data are the average of three independent infections of hepatocytes. D, hepatocytes were infected with Ad-PGC-1α at three different multiplicities of infection (MOI) as indicated above the Western blot. Proteins were prepared from whole liver extract of streptozotocin-induced diabetic and control rats. The PGC-1α abundance was determined by Western analysis, and the protein source is indicated above the immunoblot.

**FIG. 2.** Identification of the rat PDK4 promoter. A, a model of the rat PDK4 first and second exons is shown. The location of gene-specific primers (GSP2 and GSP3), which were used in the RACE reactions, is shown. The highlighted adenosine (A) at +1 indicates the transcriptional start site in exon 1. B, the sequence of the rat proximal promoter is shown. Specific protein binding sites for HNF4, FOXO1, and Sp1 are underlined. The ATG (Met), where protein translation initiates, are the final nucleotides in the sequence.
First, we generated three double-stranded oligonucleotides that represented all the sequences in the 5′-1210 region of the promoter. The ability of these oligomers to bind factors in rat liver nuclear extract was tested with gel shift mobility assays. Two oligomers containing nucleotides −1221 to −1197 and −1210 to −1184 were able to bind nuclear proteins. However, none of the DNA protein complexes was altered by an antibody to HNF4 in the gel shift assays, indicating that HNF4 did not bind in this region (data not shown). Further inspection of the promoter led to the identification of a sequence between −1115 and −1092 that contained a consensus HNF4 site. We tested this DNA element in gel shift mobility assays using rat liver nuclear extract and an antibody to HNF4 (Fig. 6A). The antibody to HNF4 shifted the DNA protein complex indicating that this element could bind HNF4. Because we could not demonstrate HNF4 binding in the −1258 to −1197 region, this observation suggested that HNF4 may interact with some factor between −1258 to −1197 to mediate a transcriptional induction. We conducted gel shift mobility assays with four oligomers representing consensus HNF4 sites in the −375 to −18 region. Two of the oligomers containing the sequences between −212/−192 and −130/−110 bound HNF4 in gel shift mobility assays (Fig. 6A). Overall, the data demonstrate that HNF4 can bind to several sites in the promoter.

To evaluate the importance of these HNF4 binding sites in the regulation of PDK4 by HNF4, we disrupted each of these sites by site-directed mutagenesis. The −2989/+87 PDK4-luc vectors with mutations in each of the HNF4 binding sites were transfected into HepG2 cells along with the expression vectors for HNF4 or PGC-1α. As can be seen in Fig. 6B, disruption of the −1115/−1093 site decreased the ability of HNF4 to stimulate PDK4 expression, indicating that this element mediates the induction by HNF4. However, disruption of the more proximal HNF4 sites did not decrease the induction by HNF4. Overall, the data indicate that HNF4 stimulates PDK4 primarily through the upstream element. In addition, we tested whether disruption of these sites would decrease the ability of HNF-4 and PGC-1α responsiveness. In these experiments, the 7-fold induction by HNF-4 was decreased to 2-fold with deletion of the sequences between −1258 and −1197 (Fig. 5). The next experiments were designed to identify binding sites for HNF-4 in the PDK4 promoter. First, we generated three double-stranded oligonucleotides that represented all the sequences in the −1258 to −1197 region of the promoter. The ability of these oligomers to bind factors in rat liver nuclear extract was tested with gel shift mobility assays. Two oligomers containing nucleotides −1221 to −1197 and −1210 to −1184 were able to bind nuclear proteins. However, none of the DNA protein complexes was altered by an antibody to HNF4 in the gel shift assays, indicating that HNF4 did not bind in this region (data not shown). Further inspection of the promoter led to the identification of a sequence between −1115 and −1092 that contained a consensus HNF4 site. We tested this DNA element in gel shift mobility assays using rat liver nuclear extract and an antibody to HNF4 (Fig. 6A). The antibody to HNF4 shifted the DNA protein complex indicating that this element could bind HNF4. Because we could not demonstrate HNF4 binding in the −1258 to −1197 region, this observation suggested that HNF4 may interact with some factor between −1258 to −1197 to mediate a transcriptional induction. We conducted gel shift mobility assays with four oligomers representing consensus HNF4 sites in the −375 to −18 region. Two of the oligomers containing the sequences between −212/−192 and −130/−110 bound HNF4 in gel shift mobility assays (Fig. 6A). Overall, the data demonstrate that HNF4 can bind to several sites in the promoter.

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Fold Induction of PDK4-luciferase

|   | HNF-4 | PGC-1α | HNF-4/PGC-1α |
|---|-------|--------|-------------|
| -578/-487 | 4.6 ± 0.2 | 2.4 ± 0.1 | 1.1 ± 0.1 |
| -504 | 3.5 ± 0.2 | 2.0 ± 0.1 | 1.1 ± 0.1 |
| -461 | 2.1 ± 0.1 | 1.1 ± 0.1 | 1.1 ± 0.1 |
| -375 | 1.1 ± 0.1 | 1.1 ± 0.1 | 1.1 ± 0.1 |

FIG. 5. Characterization of HNF-4-responsive regions in the PDK4 promoter. Serial deletions were created between −1502 and −1197 in the rPDK4 promoter. A model of the truncated promoters is shown on the left side. HepG2 cells were transfected with 2 μg of rPDK4-luciferase reporter gene and expression vectors for HNF-4 (0.25 μg), pSV-PGC-1α (1 μg), and TK-Renilla (1.0 μg) as described in the legend to Fig. 3. All transfections were done in duplicate and repeated four times.

A

| Oligomer | rPDK4-212/-192 | rPDK4-130/-110 | rPDK4-1115/-1092 |
|---------|---------------|----------------|------------------|
| HNF-4 Ab | −−−−+−−−−−++−−−−| | |
| RLNE | −−−−−−−−−−−−+−−−−|

B

| Oligomer | rPDK4-luc | HNF-4 | PGC-1α | HNF-4/PGC-1α |
|---------|----------|-------|--------|-------------|
| -2989 | 8.0 ± 0.8 | 5.8 ± 0.9 | 13.9 ± 3.4 |
| -2989 mut-1115 | 2.1 ± 0.5 | 6.2 ± 0.7 | 5.0 ± 0.3 |
| -2989 | 10.8 ± 3.4 | 4.2 ± 0.2 | 13.9 ± 2.9 |
| -2989 mut-130 | 8.3 ± 1.8 | 3.6 ± 0.1 | 12.9 ± 3.0 |

FIG. 6. Identification of HNF-4 binding sites in the PDK4 promoter. A, gel shift mobility assays were conducted to demonstrate the binding of HNF-4 to specific oligomers. The nucleotides contained in the 32P-labeled oligomer are indicated at the top. The oligomers were incubated with either rat liver nuclear extract (RLNE) or an antibody against HNF-4 (HNF-4 Ab). The resulting protein DNA complexes were resolved on a non-denaturing acrylamide gel. B, the HNF4 binding sites were mutagenized in the PDK4-luc vector. Transfections with expression vectors for HNF4 and PGC-1α were conducted as described in the legend to Fig. 3. The data are expressed as the induction by HNF4 or PGC-1α. All transfections were done in duplicate and repeated three times.

PGC-1α to induce PDK4 gene expression. The mutations did not decrease the ability of PGC-1α to induce the expression of PDK4 (Fig. 6B). The results indicate that HNF-4 does not contribute to the recruitment of PGC-1α to the PDK4 gene.

To identify which sequences in the regions −578 to −375 might be important for recruiting PGC-1α to the PDK4 gene, we generated additional deletions in this region. The deletion of nucleotides between −504/−461 and −423/−375 decreased the ability of PGC-1α to induce the PDK4 promoter, suggesting that at least two factors are recruiting PGC-1α to the gene (Fig. 7). We ligated the −578/−328 and −578/−375 regions in front of the enhancerless SV40-luc reporter gene. These vectors were transfected with either pSV or pSV-PGC-1α into HepG2 cells. As shown at the bottom of Fig. 7, deletion of nucleotides −375 to −328 greatly decreased the ability of PGC-1α to stimulate gene expression. Two copies of the −375/−328 regions were ligated in front of SV40-luciferase, and this region mediated an induction by PGC-1α. The data in this figure demonstrate that at least two regions between −578 and −328 are involved in the induction of the PDK4 gene by PGC-1α.

We conducted ChIP assays with the PDK4 promoter to test whether HNF-4 and PGC-1α were associated with the PDK4 gene in vivo. Primary rat hepatocytes were cross-linked with 1% formaldehyde, and the chromosomal DNA was sheared by repeated sonication. The DNA-protein complexes were immunoprecipitated using either rabbit IgG as a control or antibodies to HNF-4 and PGC-1α. Using primers to the −1504/−1197 region of the PDK4 gene, we observed a strong PCR product with DNA immunoprecipitated with the HNF-4 antibody but not with the PGC-1α antibody (Fig. 8). The PGC-1α antibody did immunoprecipitate some chromosomal DNA based upon the weak PCR product. On the other hand, PCR primers around the proximal promoter generated substantial PCR products with DNA immunoprecipitated by both the PGC-1α and HNF-4 antibodies. These results indicate that HNF-4 and PGC-1α are bound to the rat PDK4 promoter in vivo.

DISCUSSION

In this study we have identified new mechanisms by which PDK gene expression is induced. Our data demonstrate that PGC-1α will stimulate PDK4 and PDK2 gene expression and suggest that this induction may have an important role in gluconeogenesis. PDC catalyzes the metabolism of pyruvate to acetyl-CoA, and the activity of this enzyme controls the fate of pyruvate. PDC is inhibited by phosphorylation by the PDK enzymes. The inhibition of PDC will lead to the accumulation of pyruvate in the liver (1, 2). Pyruvate can enter the hepatic gluconeogenic pathway after conversion to oxaloacetate. The PDK4 and PDK2 genes are up-regulated in the liver of fasted and diabetic animals (4, 6, 7). Our data suggest that PGC-1α will provide gluconeogenic substrates by reducing pyruvate.
metabolism to acetyl-CoA. Our results have outlined a new mechanism by which PGC-1α contributes to hepatic gluconeogenesis and hyperglycemia observed in the diabetic phenotype.

In our studies we have provided the first cloning and characterization of the rat PDK4 promoter. The mouse and human PDK4 promoters have also been cloned (27, 28). We found with our RACE analysis that the transcriptional start site was about 50 base pairs 5′ to the previously reported 5′ end of the rat PDK4 cDNA. The rat gene is 91% homologous to the mouse gene within the first 500 base pairs of the proximal promoter, whereas the human PDK4 promoter has only a limited homology to the rat promoter. There is no TATA box in either the rat or mouse promoter (27, 28). Conserved elements include an Sp1 site, which may contribute to the basal expression. In addition, the two HNF-4 binding sites in the rat proximal promoter are perfectly conserved in the mouse gene, and we have shown that HNF4 can stimulate the mouse promoter. A binding site for forkhead transcription factor FKHL/FOXO1 has been identified in the mouse promoter at −362–355, and this site is conserved in the human and rat genes (27, 28). FOXO1 participates in the stimulation of human PDK4 by glucocorticoids (27, 28). The sequence conservation between the rodent species suggests that these factors are important regulatory factors for this gene.

Multiple lines of evidence indicate that PGC-1α will promote hepatic gluconeogenesis (10, 13, 18). Overexpression of PGC-1α will stimulate PEPCk and glucose-6-phosphatase gene transcription (10, 13, 18). Expression of the PEPCk gene is inhibited by small interfering RNA to PGC-1α (31). ChIP assays have shown that PGC-1α is associated with the PEPCk and glucose-6-phosphatase promoters (17, 31). Finally, hepatocytes prepared from PGC-1α knock-out mice have reduced PEPCk expression (32). In our studies we have found that PGC-1α will stimulate PDK4 and PDK2 gene expression, and our ChIP assays demonstrated that PGC-1α is directly associated with the rat PDK4 promoter in primary hepatocytes. When pyruvate was given to PGC-1α knock-out mice, there was reduced conversion of this pyruvate into glucose (32). This observation suggests that PGC-1α has a role in regulating pyruvate metabolism. Overall, our data support the concept that PGC-1α will stimulate multiple steps in the gluconeogenic pathway.

We have found that HNF4 will stimulate the PDK4 gene at least in the liver. HNF4 directs the expression of multiple genes in the pancreas and liver. Mutations in HNF4 are associated with maturity onset diabetes type 1 (MODY1) (33, 34). In fact, it was found that HNF4 is associated with 42% of the promoters of actively transcribed genes in human hepatocytes (30). The human PDK4 gene was among the genes that bound HNF4, although specific binding sites were not identified in the human promoter (30).

In the liver, PGC-1α stimulates PEPCk gene expression through interactions with multiple transcription factors including HNF-4, FOXO1, and the glucocorticoid receptor (10, 13, 18). Mutation of the binding sites for any of these factors decreased the induction of the PEPCk gene by PGC-1α. HNF-4 is also involved in the stimulation of the glucose-6-phosphatase gene by PGC-1α (10, 13, 18). In the PDK4 gene, the induction by PGC-1α is decreased by deletion of sequences between −504 and −328. Because the PGC-1α induction decreased with sequential deletions suggests that several factors recruit PGC-1α. However, unlike the PEPCk and glucose-6-phosphatase genes, HNF4 is not involved in the recruitment of PGC-1α. In addition, glucocorticoids do not stimulate rat PDK4 gene expression through the portion of the promoter that we cloned (data not shown). This observation suggests that the glucocorticoid receptor is not necessary for the recruitment of PGC-1α to the rat PDK4 promoter. FOXO1 may participate in the recruitment of PGC-1α since there is a FOXO1 site in the −375/−328 region that is perfectly conserved among rat, mouse, and human genes. However, it is clear that PGC-1α stimulates the PDK4 and PDK2 genes by different mechanisms.

Acknowledgment—We thank Dr. B. Spiegelman for the adenovirus expressing PGC-1α and GFP. Dr. Furuyama for the mouse PDK4-luc vector, and Keerthi G adiparthi for sequencing the RACE PCR products.

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**PGC-1α Stimulates PDK4 and PDK2**