Glucocorticoids Repress Transcription of Phosphoenolpyruvate Carboxykinase (GTP) Gene in Adipocytes by Inhibiting Its C/EBP-mediated Activation*

Received for publication, January 9, 2003
Published, JBC Papers in Press, January 30, 2003, DOI 10.1074/jbc.M300263200

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The cytosolic form of the phosphoenolpyruvate carboxykinase (PEPCK-C) gene is selectively expressed in several tissues, primarily in the liver, kidney, and adipose tissue. The transcription of the gene is reciprocally regulated by glucocorticoids in these tissues. It is induced in the liver and kidney but repressed in the white adipose tissue. To elucidate which adipocyte-specific transcription factors participate in the repression of the gene, DNase I footprinting analyses of nuclear proteins from 3T3-F442A adipocytes and transient transfection experiments in NIH3T3 cells were utilized. Glucocorticoid treatment slightly reduced the nuclear C/EBPα concentration but prominently diminished the binding of adipocyte-derived nuclear proteins to CCAAT/enhancer binding protein (C/EBP) recognition sites, without affecting the binding to nuclear receptor sites in the PEPCK-C gene promoter. Of members of the C/EBP family of transcription factors, C/EBPa was the strongest trans-activator of the PEPCK-C gene promoter in the NIH3T3 cell line. The glucocorticoid receptor (GR), in the presence of its hormone ligand, inhibited the activation of the PEPCK-C gene promoter by C/EBPa or C/EBPβ but not by the adipocyte-specific peroxisome proliferator-activated receptor-γ2. This inhibition effect was similar using the wild type or mutant GR and did not depend on GR binding to the DNA. The glucocorticoid response unit (GRU) in the PEPCK-C gene promoter (−2000 to +73) restrained C/EBPα-mediated transactivation, because mutation of each single GRU element increased this activation by 3–4-fold. This series of GRU mutations were repressed by wild type GR to the same percent as was the nonmutated PEPCK-C gene promoter. In contrast, the repression by mutant GR depended on the intact AF1 site in the gene promoter, whereby mutation of the AF1 element abolished the repression.

Glucocorticoids play a fundamental role in the maintenance of homeostasis in mammals. Removal of the adrenals severely compromises the ability of animals to withstand fasting (for reviews see Refs. 1 and 2). Glucocorticoids exert their effects via the glucocorticoid receptor (GR), predominantly by modulating gene transcription (3–5). An attractive mode of regulation, especially in light of the coordinated effects of glucocorticoids in maintaining homeostasis, is the opposing control of the same gene in different tissues by GR. PEPCK-C gene expression was the strongest inhibition effect in the liver and kidney (6, 7) but is repressed in the adipose tissue (8). PEPCK-C catalyzes a key reaction that determines the rates of gluconeogenesis in the liver and kidney and glyceroneogenesis in the adipose tissue and liver (9). Glyceroneogenesis, the de novo synthesis of 3-glycerophosphate from pyruvate and amino acids (via an abbreviated version of gluconeogenesis), provides this precursor for the synthesis of triglycerides (10, 11). Recently, we have performed a targeted mutation in the adipose tissue-specific enhancer of the PEPCK-C gene in embryonic stem cells. The mutation ablated PEPCK-C gene expression in white adipose tissue of mice homozygous for this mutation and caused a decrease in the storage of triglycerides, which in some mice developed into lipodystrophy (12). This mutation therefore established the importance of PEPCK-C and glyceroneogenesis in the homeostasis of triglycerides in the adipose tissue.

Because PEPCK-C is encoded by a unique copy gene, and is transcribed from a single promoter, it is likely that tissue-specific factors are involved in the reciprocal regulation that leads to stimulation (liver and kidney) or repression (adipose tissue) of the gene transcription in the presence of glucocorticoids. Yamamoto and colleagues (13) proposed the term composite GRE to describe a nonconsensus sequence that binds the GR with low affinity and, in turn, is capable of mediating either repression or activation of genes. The GRE identified in the PEPCK-C gene (14) is a nonconsensus sequence that binds GR at a very low affinity and is not able by itself to transmit a transcriptional response to glucocorticoids. In fact, PEPCK-C gene promoter harbors GRU containing two low affinity, nonconsensus sequences that bind GR at a very low affinity and are not able by itself to transmit a transcriptional response to glucocorticoids.

The abbreviations used are: GR, glucocorticoid receptor; PEPCK-C, phosphoenolpyruvate carboxykinase-C; C/EBP, CCAAT/enhancer-binding protein; GRE, glucocorticoid-response element; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; CRE, cyclic AMP-response element; PPARe, PPAR-response element; RTR, reverse transcriptase; CAT, chloramphenicol acetyltransferase; GRU, glucocorticoid response unit; DMEM, Dulbecco's modified Eagle's medium; HNF, hepatocyte nuclear factor; COUP-TF, chicken ovalbumin upstream transcription factor.

This paper is available on line at http://www.jbc.org

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* This work was supported by Grants 1999348 and 8690117 from the United States-Israel Binational Science Foundation, Grant 540197-19 from the Israel Science Foundation, a grant from the Israeli Ministry of Health, and by Grant DE22541 from the National Institutes of Health.

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ments do not bind steroid receptors, but their occupancy is required for the response of the PEPCK-C gene to glucocorticoids (see scheme in Fig. 2). The factors binding to the AF1 element are all nonsteroid nuclear receptors; these include hepatocyte nuclear factor (HNF) 4, chicken ovalbumin up-stream transcription factor (COUPTF), retinoic acid receptor, retinoid X receptor (RXR), and members of the peroxisome proliferator-activated receptor (PPAR) family. The AF2 site (15) binds HNF3β (16, 17) and has been proposed to comprise an insulin-response element as well (18).

To identify tissue-specific factors that are involved in the glucocorticoid-mediated repression of PEPCK-C gene transcription in adipocytes, we have employed a systematic DNase I footprinting analysis of the PEPCK-C gene promoter, using adipocyte nuclear proteins. Their functional participation in the repression has been assessed using transient transfection experiments in PEPCK-nonexpressing NIH3T3 cells. The results from these two independent experimental systems consistently identified the involvement of members of the C/EBP family, but not those of PPAR, in the GR repression of the PEPCK-C gene promoter activity. Furthermore, experiments in NIH3T3 cells revealed a hierarchical constraint of PEPCK-C gene promoter trans-activation by the separate GRU elements. Both wild type and mutant GR (incapable of binding the DNA) repress the C/EBP-mediated trans-activation by 50–60%, regardless of whether the trans-activation is low or high. However, the repression by mutant GR critically requires an intact AF1 site in the PEPCK-C gene.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium (DMEM), F-12, and fetal and newborn calf serum were purchased from Biological Industries, Kibbutz Beit Haemek, Israel. Biosynthetic human insulin was obtained from Novo Nordisk (Denmark). Dexamethasone, the synthetic glucocorticoid hormone, was purchased from Teva, Israel Pharmaceutical Industry, Ultraspec, the commercial reagent for the preparation of tissue RNA, was purchased from Biotec Laboratories, Inc. (Austin, TX). Radioactive signals were quantified using a PhosphorImager (Fuji BAS 1000, Fujifilm, Japan). Reverse transcription was obtained from Invitrogen. Random hexanucleotide pd(N)₆ was purchased from Amersham Biociences, and the ribonuclease inhibitor, RNasin, was purchased from Promega (Madison, WI). The enzyme-linked immunosorbent assay kit for the determination of human somatotropin was purchased from RIA-BioSource (Camarillo, CA). Dexamethasone treatment—3T3-F442A cells, obtained from Dr. Howard Green (19), were grown to confluency in DMEM and supplemented with 10% newborn calf serum. For differentiation of the cells to adipocytes, newborn calf serum was replaced by 10% fetal calf serum; isobutylmethylxanthine was added at a final concentration of 0.2 mM, and the cells were incubated for 3 days. The cells were further incubated for at least 5 days in a medium containing 4 milliunits/ml insulin, until ~80–90% of cells contained fat droplets.

**DNA Isolation and RT-PCR Analysis**—Total RNA was isolated from a single 100-mm cultured plate of 3T3-F442A adipocytes or from 30 mg of mouse liver using the commercial reagent Ultraspec according to the manufacturer’s instructions. One μg of total RNA was reverse-transcribed using the manufacturer’s protocol, the reverse transcriptase kit in the presence of 7.5 units/ml random hexanucleotide pd(N)₆ as primer and 1 unit/ml RNasin ribonuclease inhibitor, except that the incubation was for 60 min at 42 °C.

PCR was performed using the PEPCK-C primers 5′-CTTGTCTAC-GAAGCTTCAG from exon 9 and 3′-CGTCGGAGACATCCCCTC from exon 11. Primers for the AF2 gene were 5′-CCTGGAAGCTGGCTTC- CAG from exon 1 and 3′-CTTGTCTGGAAGATCGGCC from exon 4. Primers for β-actin were the same as published previously (20). PCR was performed in the presence of a trace of [32P]dCTP (0.5 × 10⁶ dpm) to allow semi-quantification (20). The PCR program included denatur-ation at 94 °C for 1 min; annealing at 55 °C for 1 min, and elongation at 72 °C for 2 min, 20 cycles each consisting of denaturation for s, annealing for 1 min, and elongation for 1 min. The PCR product was separated by electrophoresis on 8% polyacrylamide gel and quantified using a PhosphorImager apparatus and visualized by its exposure to autoradiographic film.

**Preparation of Nuclear Proteins**—Nuclear proteins were extracted from rat liver according to Gorski et al. (21) as modified (22). Nuclear protein extracts from adipocytes were prepared essentially as described previously (22), except that the sucrose gradient step to further purify the nuclei was omitted. DNase I footprinting assays were performed as described previously (22). The autoradiographic density signals of specific bands in the exposed film (see Figs. 2–4) were quantified using FunScan-MultiAnalyst with version 1.1 (Bio-Rad).

**Western Blot Analysis**—5 μg of nuclear proteins from adipocytes treated or untreated with dexamethasone (a synthetic glucocorticoid) were separated on 15% SDS-PAGE and transferred to nitrocellulose membrane (Protran BA 85, Schleicher & Schuell). C/EBPα was probed with rabbit polyclonal anti-C/EBPα antibody c100 diluted 1:1000 (a gift from Dr. Steven McKnight) and was detected using horseradish peroxidase-conjugated goat anti-rabbit antibody diluted 1:4000. Nuclear Y12 protein was probed with mouse anti-sm monoclonal antibody Y12 (23) (a gift from Dr. Ruth Sperling), diluted 1:10, and detected using horseradish peroxidase-conjugated goat anti-mouse IgG (Fab/ fragment diluted 1:3000 (23). Secondary antibodies were visualized with SuperSignal West Pico chemiluminescent substrate (Pierce).

**Cell Culture, Transfection Conditions, and CAT Assays**—NIH3T3 cells were grown on 100-mm plates in DMEM containing 10% fetal calf serum. For transfection, cells were transferred to DMEM containing 10% newborn calf serum. Transfection was performed essentially according to Chen and Okayama (24), as described previously (25), 1 or 2 days after the cells reached confluence. Supernatant from CAT plasmid (2 μg) and additional carrier pBlueScript DNA (Stratagene), to make a total of 12 μg, were used, and the transfection efficiency was monitored as described previously (25). Where indicated, 1 μg each of the expression vectors for C/EBPα and PPARγ2 together with RXRα or GR was used. The optimal quantity of C/EBPβ or C/EBPδ expression vector required for half-maximal repression was 0.5 μg. A titration test of various concentrations of the expression vectors allowed us to choose the amounts that yielded optimal effects. Assays for chloroamphenicol acetyltransferase (CAT) activity were determined 44 h after transfection, as described (25), and quantified using a PhosphorImager apparatus. The S.E. was calculated as the S.D. divided by the square root of the number of experiments.

**Plasmids Used in the Transfection Studies**—The previously described plasmid 597-pck-CAT (PCK(597)-CAT) contains 597 bp of the rat PEPCK-C gene promoter region fused to the CAT reporter gene (26). The derived 2000-pck-CAT plasmid (PCK(2000)-CAT) contains 2000 bp of the rat PEPCK-C gene promoter (27). The mutated AF1 (AF1-mut), the combined mutation of GRE1 and GRE2 (mGRE1–2) (28), and the mutated AF2 (AF2-mut) (29) plasmids contain 2000 bp of the rat PEPCK-C gene promoter mutated at these sites. The mouse promoters PCK(840)-CAT and PCK(1500)-CAT contain 840 or 1500 bp upstream from the transcription start site of PEPCK-C gene promoter, derived from a mouse genomic clone, and fused to the CAT reporter gene as described previously (30). The PCK(1500)-mut1-CAT plasmid contains a site-specific mutation of the PPARE sequence. The mutation was generated by inserting the restriction sites Xhol and Smal 3′ and 5′, respectively, to the PPARE site. This enabled us to replace the PPARE site with 47 bp of the pBlueScript polylinker residing between Xhol and Smal, except that the linker insert was deleted.

The DNA concentration of the constructs containing the longer PEPCK-C gene promoters was corrected to achieve the same number of molecules as that of the shorter PEPCK-C gene promoters. Expression vectors encoding adipocyte-enriched transcription factors used in this work included the following: C/EBPα (30) obtained from Dr. Steven McKnight; C/EBPβ (31) from David Ron; C/EBPγ (32) from Dr. Daniel Lane; PPARγ2 (33) from Dr. Bruce Spiegelman; RXRα (34) and human GR (35) from the laboratory of Dr. Ron Evans; and rat wild type and mutant GR were a gift from Dr. Keith Yamamoto (13).

**RESULTS**

**Repression of PEPCK-C Expression by Glucocorticoids in Vivo**—The addition of glucocorticoids to fully differentiated 3T3-F442A adipocytes for 16–18 h caused a strong repression of PEPCK-C gene expression as shown by the absence of its RT-PCR product (Fig. 1). This repression was distinct because...
Glucocorticoid-induced transcription factors are known to bind specific DNA sequences, and these sequences are often referred to as glucocorticoid response elements (GREs) or glucocorticoid response elements (GREs). These elements are typically located in the promoter region of genes and are involved in the regulation of gene expression by glucocorticoids.

In the context of the PEPCK-C gene promoter, glucocorticoids failed to inhibit the expression of an adipocyte-specific aP2 gene (Fig. 1).

**DNase I Footprinting Assays**—In order to assess whether GR affects the binding of nuclear proteins to specific sites in the PEPCK-C gene promoter, we systematically footprinted the gene promoter, using nuclear proteins extracted from 3T3-F442A adipocyte cells that had been incubated without or with glucocorticoids. A scheme of PEPCK-C gene promoter depicting the binding sites of transcription factors is shown (Fig. 2a). The figure also includes the binding sites for nuclear proteins present in the liver, fetal liver (where the gene for PEPCK-C is not actively transcribed [22, 36]), kidney, and adipocytes (Fig. 2b).

Note that nuclear proteins from the liver and adipocytes bind to most sites included within positions −70 to −1200 of the transcription start site of the PEPCK-C gene promoter. Adipocyte nuclear proteins do not bind to the P2 site (HNF-1 recognition motif) in the PEPCK-C gene promoter (37), whereas nuclear proteins from the liver bind poorly to PPAR (the PPAR recognition site) (see Fig. 3b). Unlike the kidney and fetal liver, nuclear proteins from adipocytes and liver bind all C/EBP recognition sites. These include the CRE-1 (cAMP-response element) P3I, P4, and CRE-2 sites (CRE-2 is a CRE-like sequence). Of these, P3I binds exclusively isoforms of the C/EBP family (38). CRE-1 and P4 sites bind AP1 as well, and CRE-1 also binds cAMP-response element-binding protein, ATF-2 (39), and ATF-3 (40). CRE-2 binds C/EBPα with very low affinity but binds a nonidentified temperature-labile protein in the liver nuclear proteins (22).

Our footprinting analysis revealed that nuclear proteins extracted from glucocorticoid-treated adipocytes failed to bind to all C/EBP recognition sites in the PEPCK-C gene promoter: CRE-1 (Fig. 2c), P3 and P4 (Fig. 2d), and the single C/EBP-like CRE-2 site (Fig. 2c). The prominent inhibition of binding to all these sites occurred despite the wide spectrum of affinities of the C/EBP recognition sites to C/EBPα, which gradually decreases from the highest affinity (CRE-1 site) to the lowest (CRE-2 site) (22, 41). In addition, binding to the P1 site (nuclear factor 1 recognition site) in the PEPCK-C gene promoter was also inhibited (Fig. 2c), although this is not a C/EBP recognition site. In previous studies (38) the binding of C/EBPβ to CRE-1 has been shown to cooperate with the binding to the P1 site. Therefore, a reduced binding to CRE-1 site might have led to a secondary effect on the binding to the P1 site.

The binding of adipocyte nuclear proteins to the recognition sites of nuclear receptors in the PEPCK-C gene promoter has not been affected by the glucocorticoid treatment. Thus, binding to AF1 site (also termed P6) remained similar whether using nuclear proteins from adipocytes not treated or treated with glucocorticoids (Fig. 3a). AF1 site is a nuclear receptor recognition site that interacts with a variety of nonsteroid nuclear receptors including HNF4, COUPTF, retinoic acid receptor, RXR, and members of the PPAR family. Similar to adipocytes, hepatic nuclear proteins also interact well with the AF1 site (Fig. 3a).

Another non-C/EBP-binding site, which likewise has not been affected by the glucocorticoid treatment, is PPAR (the recognition site of the heterodimer nuclear receptors PPAR2/ RXR) (Fig. 3b). In contrast to the adipocyte nuclear proteins, binding of hepatic nuclear proteins to the PPAR site could barely be detected (Fig. 3b), unlike their efficient binding to the AF1 site (Fig. 3a). Furthermore, the hypersensitive site (position −985) at the 3′ end of the protected region appeared only in the presence of adipocyte nuclear proteins. It was undetectable both in the absence of nuclear proteins and in the presence of liver nuclear proteins (Fig. 3b). Therefore, PPAR isoforms seem less enriched in the liver (from nonfasted rat) than they are in adipocyte nuclear proteins. To gain quantitative estimation of the glucocorticoid effect, we measured the density signals of specific bands inside and outside the protected regions of the CRE-1 (Fig. 2a) and AF1 (Fig. 3a) sites. The footprinting intensity of the CRE-1 site was quantified from the autoradiographic films by measuring the density signal of a band within the CRE-1 site (position −88 from the transcription start site of the CRE-1 site was quantified from the autoradiographic films by measuring the density signal of a band within the CRE-1 site (position −88 from the transcription start site of the CRE-1 site was quantified from the autoradiographic films by measuring the density signal of a band within the CRE-1 site (position −88 from the transcription start site of the CRE-1 site was quantified from the autoradiographic films by measuring the density signal of a band within the CRE-1 site (position −88 from the transcription start site of the CRE-1 site was quantified from the autoradiographic films by measuring the density signal of a band within the CRE-1 site (position −88 from the transcription start site of the CRE-1 site was quantified from the autoradiographic films by measuring the density signal of a band within the CRE-1 site (position −88 from the transcription start site of
the PEPCK-C gene) and a band outside, at position −160 (both marked by arrows). Likewise, the footprinting intensity of the AF1 site was quantified by measuring the density signals of two bands inside (positions −445 and −446) and a band outside (position −457) the AF1 site. The ratios between the density signals inside and outside the protected region were computed. The ratios obtained from the footprinting done without nuclear proteins were arbitrarily set at 10 and used to normalize other ratios of the footprinting done in the presence of nuclear proteins. These measurements have clearly assessed that dexamethasone treatment interfered with the adipocyte nuclear protein footprinting of the CRE-1 site (Fig. 4c) but not with the footprinting of the AF1 site (Fig. 3d).

Transcription of the gene for C/EBPα is also repressed by glucocorticoids but only for a few hours (32). Yet this repression might lead to a longer lasting reduction of C/EBPα protein concentration in adipocyte nuclei (32), resulting in an apparent, rather than real, interference of binding to its recognition sites. Therefore, we determined whether the concentration of C/EBPα in nuclei corresponded to the observed diminished footprinting of the PEPCK-C gene promoter.

C/EBPα Level in Extracted Adipocyte Nuclear Proteins—The concentration of C/EBPα in the adipocyte extracts of nuclear proteins used for footprinting was determined by Western blot assay. The hormonal treatment diminished the nuclear concentration of C/EBPα by 30% when normalized to the level of the Y12 nuclear protein (Fig. 4a). Whether this reduced concentration of C/EBPα accounted for the inhibited footprinting of nuclear proteins from glucocorticoid-treated adipocytes was assessed. Thus, we used lower amounts of nuclear proteins extracted from untreated adipocytes (10 (2/3) and 7.5 μg (1/2)), compared with the whole amount (15 μg), to footprint the gene promoter region containing the CRE-1, P1, and CRE-2 sites. This region enabled us to assay the site with the highest affinity for C/EBP binding (CRE-1) and the site with the lowest affinity for C/EBP binding (CRE-2). The analysis showed that 10 μg of nuclear proteins footprinted all three sites (Fig. 4b). Footprinting of the CRE-1 site was quantified as detailed above for Fig. 2c. Thus, the ratio in the absence of proteins was set at 10, relative to a ratio of 2.5 obtained with 10 μg of protein and a ratio of 8.5 obtained with 7.5 μg of protein (Fig. 4c). The dexamethasone effect on CRE-1 footprinting (Fig. 2c) that was likewise quantified yielded a ratio of 1.7 in the presence of 15 μg of nuclear proteins from untreated adipocytes, and 15.8 in the presence of 15 μg of nuclear proteins from dexamethasone-treated adipocytes (Fig. 3c). In contrast, footprinting the AF1 site yielded similar ratios (2.7 and 3.3, respectively) using 15 μg of nuclear proteins from untreated or dexamethasone-treated adipocytes (Fig. 3d). We therefore conclude that in addition to lowering the level of C/EBPα in the nucleus, the hormonal treatment interfered with the binding to their recognition sites in the PEPCK-C gene promoter.

Transient Transfection Experiments in NIH3T3 Cells—The involvement of adipocyte-enriched transcription factors in the repression of PEPCK-C gene transcription by glucocorticoids was further assessed using transient transfection assays in NIH3T3 cells. Although these cells do not express PEPCK-C or the adipocyte-specific transcription factors PPARγ2 or C/EBPα (42), co-transfecting expression vectors coding for PPARγ2 and RXRα (PPARγ2/RXR) were reported to stimulate transcription from the PEPCK-C gene promoter in these cells (33). We have compared the stimulation of the PEPCK-C gene promoter activity by PPARγ2/RXR and by C/EBPα’s expression vectors using PEPCK-CAT chimeric genes driven either by 600 (PCK(600)-CAT) or 2000 bp (PCK(2000)-CAT) of the PCK(2000)-CAT gene promoter (27). PPARγ2/RXR preferentially stimulated transcription from the PCK(2000)-CAT PEPCK-C gene promoter, whereas C/EBPα’s preferentially and markedly stimulated transcription from the PCK(600)-CAT gene promoter (Fig. 5a). The latter is most likely because of the localization of a cluster of C/EBP recognition sites in the proximal region of the PEPCK-C gene promoter (9). For orientation see the scheme of the

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**Fig. 3.** DNase I footprint analysis of the nuclear receptor-binding sites in the PEPCK-C gene promoter. a, a 128-bp DNA fragment, spanning positions −490 to −342 of the rat PEPCK-C gene, was 32P-end-labeled at the 5′ site (position −490) of the fragment. 50,000 cpm of the labeled probe was added per reaction. Incubation without (0′) or with 15 μg of nuclear proteins from 3T3-F442A adipocytes not treated (A − Dex), or treated overnight (A + Dex) with dexamethasone or with 10 μg of rat liver nuclear proteins (L) are indicated. The lane on the right (seq) designates sequencing of A + G, which enabled the identification of the region protected by proteins binding to the AF1 site. The protected region is marked by the positions 5′ to the transcription start site, b, a 160-bp DNA fragment, spanning positions −550 to −1150 of the rat PEPCK-C promoter, was 32P-end-labeled at the 3′ site (position −950) of the fragment. 50,000 cpm per reaction of the labeled fragment were incubated without proteins (0′) or with 15 μg of nuclear proteins from 3T3-F442A adipocytes not treated (A − Dex) or treated overnight (A + Dex) with dexamethasone or with 10 μg of rat liver nuclear proteins (L). The lane on the right (seq) designates sequencing of A + G, which enabled the identification of the region protected by proteins binding to PARE site, indicated by the positions 5′ to the transcription start site, c, the ratio between the density signal of a distinct band within the protected CRE-1 site (position −88) and a band outside (position −160) (marked by arrows in Fig. 2b) are shown in the histogram. The ratio in the absence of proteins was set at 10. d, the ratio between the density signals of two distinct bands within the AF1 site-protected region (positions −445 and −446) and a band outside (position −457) (marked by arrows in a) is shown by the histogram. The ratio in the absence of proteins was set at 10.

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Regulation of PEPCK-C Gene Transcription in Adipose Tissue

PEPCK-C gene promoter (Fig. 2a). In addition to C/EBPα, other members of the C/EBP family also trans-activated the PEPCK-C gene promoter but to a lesser extent. C/EBPβ was the most effective; C/EBPδ was half as effective, and C/EBPδ was the least effective. There was a 5-fold difference in the magnitude of stimulation between C/EBPδ and C/EBPδ (Fig. 5b). We then determined the effect of GR and its hormone (dexamethasone) on the level of trans-activation of the PCK(6000)-CAT gene promoter by co-transfected C/EBPα, C/EBPδ, or PPARγ2/RXR. Co-transfection of GR expression vector and the addition of dexamethasone for the last 24 h after transfection inhibited (by about 60%) the activation of transcription from the PEPCK-C gene promoter either by C/EBPα or by C/EBPδ. In contrast, there was no effect of GR and dexamethasone on the trans-activation by PPARγ2/RXR of either PCK(6000)-CAT (Fig. 5c) or PCK(2000)-CAT (Fig. 5d) gene promoters.

The preferential PPARγ2/RXR trans-activation of the longer region of the PEPCK-C gene promoter, either PCK(2000)-CAT (rat) or PCK(1500)-CAT (mouse) gene promoters compared with PCK(6000)-CAT (rat) and PCK(8000)-CAT (mouse) (Figs. 5a and 6a), is due to the two PPARγ2/RXR recognition sites (a proximal AF1 site and a distal PPAR site). Moreover, mutation of the PPARα sequence in PCK(1500)-CAT gene promoter (mouse PCK(1500-mut)-CAT) abolished its response to PPARγ2/RXR, although it contained the proximal AF1-binding site (Fig. 6a). Note that the sequence of PEPCK-C gene promoter is highly preserved between the rat (43) and the mouse (44). Furthermore, mutation of the PPARα sequence specifically ablated PEPCK-C gene expression in the adipose tissue in vivo as shown with the mutated rat PEPCK-C transgene in transgenic mice (45) and the targeted mutation in the mouse endogenous PEPCK-C gene (12). In contrast, mutation of the proximal PPARγ2/RXR-binding site (AF1 site) in the context of PCK(2000)-CAT gene promoter markedly increased the PPARγ2/RXR trans-activation of the gene promoter (Fig. 6b). The constraining features of the wild type AF1 site of the PEPCK-C gene promoter in NIH3T3 cells have been studied recently in detail by Eubank et al. (46). These authors showed that the AF1 site tightly binds the nuclear receptor COUP/TII whose expression vector inhibited the PPARγ2/RXR-mediated trans-activation of the PEPCK-C gene promoter via the AF1-binding site (46). These features of AF1 site are not shared by other GR elements, because mutation of the AF2 site had no effect on the trans-activation of PCK(2000)-CAT by PPARγ2/RXR (Fig. 6b).

C/EBPα-mediated trans-activation of the PCK(2000)-CAT chimeric gene is also constrained (Fig. 6c). However, unlike the trans-activation by PPARγ2/RXR, in this case mutations of any single element of the GRU (14), AF1, AF2, and GREA1–2 of PCK(2000)-CAT chimeric gene, enhanced the C/EBPα-mediated trans-activation of the gene promoter by 3- to 4-fold (Fig. 6c). Therefore, unlike PPARγ2/RXR, not only the AF1 site but the entire GRU domain constrains the C/EBPα trans-activation of
experiments. 

2000 activation by PPAR (AF2-mut), AF1 (AF1-mut), and AF2 (AF2-mut) sites in the context of the S.E. for at least nine independent experiments. b and c. Finally, we have assessed whether the enhanced C/EBPα-mediated transactivation of the PCK(2000)-CAT rat PEPCCK-C gene promoter by C/EBPα. a, effect of wild type rat GR (wt GR) and rat mutant GR (mut GR) in the presence of dexamethasone (as described in the legend of Fig. 5c) on the trans-activation of the PCK(2000)-CAT rat PEPCCK-C gene promoter by C/EBPα. The fold stimulation by C/EBPα over basal transcription activity of the PCK(2000)-CAT promoter, taken as one, represents the mean ± S.E. for at least eight independent experiments. b, effect of wild type GR (wt GR) and mutant GR (mut GR) on the trans-activation of the PCK(2000)-CAT rat PEPCCK-C gene promoter and its derived series of the GRU mutants described in Fig. 5c. The values are expressed as percent repression by GR from the C/EBPα-stimulated activity of each gene promoter. These represent the mean ± S.E. of at least four independent experiments.

GR-mediated repression of C/EBPα trans-activation of the PEPCCK-C gene promoter. When AF1 is mutated, the repression by mutant GR is abolished (Fig. 7b).

DISCUSSION

Our results shed light on the involvement of transcription activators from the C/EBP family, but not those from the PPAR family, in the glucocorticoid-mediated repression of PEPCCK-C gene transcription in adipocytes. Glucocorticoid treatment of 3T3-F442A adipocytes led to a reduced nuclear concentration of C/EBPα, in addition to a markedly diminished binding of nuclear proteins to the C/EBP recognition sites (but not to the PPAR/RXR recognition sites) in the PEPCCK-C gene promoter. Previous studies from our laboratory (26) documented a requirement for the C/EBP recognition sites of the PEPCCK-C gene promoter that were crucial for its basal activity in adipocytes. Transient transfection in NIH3T3 cells revealed that GR together with glucocorticoids partially inhibited the C/EBPα-mediated, but not PPARγ2-mediated, trans-activation of PEPCCK-C gene promoter. We have thus described the involvement of members of the C/EBP family in the adipocyte-specific glucocorticoid repression of PEPCCK-C gene expression. This occurs in at least two ways: (a) by reducing the concentration of C/EBPα in adipocyte nuclei, and (b) interfering with the binding of nuclear proteins to the C/EBP recognition sites in the DNA and as a consequence of, or in addition to, inhibiting the trans-activation of PEPCCK-C gene promoter by C/EBPα isoforms. Clearly, using nuclear proteins from the PEPCCK-expressing adipocytes and transient transfection in the PEPCCK-nonexpressing NIH3T3 cells comprised two independent experimental approaches and systems, both of which consistently revealed the involvement of C/EBP but not PPAR activators in the glucocorticoid repression of PEPCCK-C gene transcription. However, because GR succeeded to inhibit the C/EBP trans-activation only by 60%, it is likely that additional factors beside members of C/EBP family participate in PEPCCK-C gene transcription in the adipocytes.

Experiments using NIH3T3 cells have disclosed a hierarchical regulation of PEPCCK-C gene transcription, in particular the constraint on the gene promoter response to C/EBPα-mediated activation by the GRU element in the PEPCCK-C gene promoter. Mutating any single element within the GRU relieves this constraint, and the trans-activation by C/EBPα is markedly

FIG. 6. Effects of site-specific mutations on the trans-activation of PCK(2000)-CAT rat PEPCCK-C gene promotor. a, fold trans-activation by PPAR/2/RXR (dark bars) over basal activity (open bars) of the mouse PCK(840)-CAT (840) and PCK(1500)-CAT (1500) PEPCCK-C gene promoters and of the PCK(1500)-CAT PEPCCK-C gene promoter containing a mutation of the PPARE site (1500-mut) is shown. The fold stimulation by PPAR/2/RXR over basal transcription activity of the PCK(840)-CAT gene promoter, taken as one, represents the mean ± S.E. for at least nine independent experiments. b, effect of mutations of the AF1 (AF1-mut) and AF2 (AF2-mut) sites in the context of the PCK(2000)-CAT rat PEPCCK-C gene promoter on its trans-activation by PPAR/2/RXR (dark bars) over basal activity (open bars). The fold stimulation by PPAR/2/RXR over basal transcription activity of the PCK(2000)-CAT gene promoter (2000), taken as one, represents the mean ± S.E. for at least six independent experiments. c, effect of mutations of GRE1 and GRE2 (mGRE1-2), AF1 (AF1-mut), and AF2 (AF2-mut) sites in the context of the PCK(2000)-CAT rat PEPCCK-C gene promoter on its trans-activation by C/EBPα (hatched bars) over basal activity (open bars). The fold stimulation by C/EBPα over basal transcription activity from the PCK(2000)-CAT chimeric gene (2000), taken as one, represents the mean ± S.E. for at least four independent experiments.

FIG. 7. Effect of rat wild type and mutant GR on the trans-activation of the PCK(2000)-CAT rat PEPCCK-C gene promotor by C/EBPα. a, effect of wild type rat GR (wt GR) and rat mutant GR (mut GR) in the presence of dexamethasone (as described in the legend of Fig. 5c) on the trans-activation of the PCK(2000)-CAT rat PEPCCK-C gene promoter by C/EBPα. The fold stimulation by C/EBPα over basal transcription activity of the PCK(2000)-CAT promoter, taken as one, represents the mean ± S.E. for at least eight independent experiments. b, effect of wild type GR (wt GR) and mutant GR (mut GR) on the trans-activation of the PCK(2000)-CAT rat PEPCCK-C gene promoter and its derived series of the GRU mutants described in Fig. 5c. The values are expressed as percent repression by GR from the C/EBPα-stimulated activity of each gene promoter. These represent the mean ± S.E. of at least four independent experiments.

The PCK(2000)-CAT chimeric gene. Because each of the elements comprising the GRU constrained the C/EBPα trans-activation of the PCK(2000)-CAT chimeric gene, we asked whether-binding of GR to the PEPCCK-C gene promoter is required for its mediated repression. To this end, the capacity of the wild type rat GR was compared with its counterpart rat mutant GR that is incapable of binding to the DNA (13, 47) to inhibit the C/EBPα-mediated trans-activation of PCK(2000)-CAT. The data showed that either wild type or mutant rat GR equally repressed the stimulation of transcription from the PEPCCK-C gene promoter by C/EBPα (Fig. 7a).

Finally, we have assessed whether the enhanced C/EBPα-mediated transcriptional stimulation of the GRU-mutated series of PCK(2000)-CAT could be repressed by GR and whether the mutant GR was also effective. The wild type GR repressed the C/EBPα-mediated stimulation of the GRU series of PCK(2000)-CAT mutants to a similar extent as that of the wild type (about 50%) (Fig. 7b). The mutant GR repressed the stimulation of the wild type and GRE1–2 mutant of PCK(2000)-CAT to a similar extent, less so the AF2 mutant, but completely failed to repress the stimulation of PCK(2000)-CAT AF1 mutant (Fig. 7b). Therefore, the AF1 site is required for the repression by mutant GR but not by the wild type GR (Fig. 7b). In fact, the AF1 site emerges as an inherent constraining element on transcription from the PEPCCK-C gene promoter because it markedly restrains the trans-activation of PCK(2000)-CAT gene promoter activity either by PPARγ2 or by C/EBPα (Fig. 6, b and c). Moreover, intact AF1 is also required for the mutant


elevated. This is different from trans-activation by PPARγ/RXR, which is also constrained, but in this case, it is not exerted via each element of the GRU but exclusively by the AF1 site. In that sense, the AF1 site has emerged as a unique element whose mutation elevates by 4-fold the response of PCK(2000)-CAT to either PPARγ/RXR or C/EBPα. These constraining features of AF1 are accentuated by the very modest C/EBPα ported by the fact that the percent repression by GR of the transcription via protein-protein interactions. This notion is sup-

activation does not require DNA binding of the receptor, the docking role to facilitate the mutant GR-mediated repression of the target genes and did not necessarily involve binding to the DNA. A GR mutated in the zinc finger (13), making it incapable of binding the DNA, was as active as wild type in repressing the C/EBPα stimulation. Subsequently, we have assessed that the wild type GR equally repressed the wild type PCK(2000)-CAT and derived mutated GRU series, whereas the mutant GR repressed the mutated GRE1–2 sites and, to a lesser extent, the mutated AF2 site of PCK(2000)-CAT. However, mutation of the AF1 site abolished the capability of the mutant GR to repress the C/EBP-mediated trans-activation. Because the mutated AF1 site did not hinder the repression by wild type GR, we suggest that the AF1 site undertakes a docking role to facilitate the mutant GR-mediated repression of the PCK(2000)-CAT gene promoter. Thus, these observations expand the hierarchy of regulation to strongly suggest that, in addition to its constraint features, the wild type AF1 site is crucial for the hormonal repression.

Because the repression by GR of the C/EBP-mediated trans-activation does not require DNA binding of the receptor, the GR probably inhibits trans-activation of PEPCK-C gene transcription via protein-protein interactions. This notion is supported by the fact that the percent repression by GR of the C/EBPα-mediated activation of transcription remained 50–60% whether the activation was low (wild type PCK(2000)-CAT gene promoter) or elevated by 3–4-fold (GRU-mutated series of PCK(2000)-CAT gene promoter).

Evidence of cross-talk between members of the C/EBP family and GR has been documented in numerous systems. Members of the C/EBP family have been shown to bind directly to the ligand binding domain of a number of nuclear receptors, including GR (see Refs. 48 and 49 and for review see Refs. 5 and 50). These interactions resulted either in induction or inhibition of the target genes and did not necessarily involve binding of GR to the DNA (48, 49). Alternatively, binding of C/EBPβ to the DNA binding domain of GR has been implicated recently in the GR repression of the vitellogenin gene transcription in hepatocytes from the rainbow trout. The vitellogenin gene promoter lacks a GR-binding site (51).

Beyond the molecular aspects of the glucocorticoid repression of PEPCK-C gene transcription in adipose tissue, its physiological significance has gained new relevance. Metabolic studies have recently documented very substantial rates of glyceroneogenesis in the liver of both rats and humans during fasting (52, 53) or after ingestion of a diet high in protein but devoid of carbohydrate (52). These findings shed new light on the metabolic significance of the reciprocal control of PEPCK-C gene transcription by glucocorticoids. PEPCK-C activity catalyzes the rate-limiting step of both gluconeogenesis and glycero- neogenesis, hence regulating both pathways. This has been verified by deletion of the PEPCK-C gene in mice, resulting in neonatal lethality from hypoglycemia (54).

In addition, a targeted mutation of the adipose tissue-specific PPARγ2-binding site of the PEPCK-C gene promoter, which selectively ablates gene expression in white adipose tissue, caused a marked diminution of glyceroneogenesis in this tissue. Mice homozygous for this mutation lost lipid from adipose tissue even to the extent of lipodystrophy, attesting to the metabolic significance of PEPCK-C and glyceroneogenesis in the adipose tissue (12). Moreover, a recent adipocyte-specific knockout of glucose transporter 4, required for glucose metabolism in the adipose tissue, generated mice exhibiting features of noninsulin-dependent diabetes mellitus without a loss of triglycerides from adipose tissue (55). Finally, Franckhauser et al. (56) overexpressed a chimeric gene containing the PEPCK-C structural gene linked to the aP2 promoter in mice resulting in obesity in adult mice. Taken together, these results further emphasize the crucial role of PEPCK-C and glyceroneogenesis in maintaining lipid homeostasis in the adipose tissue. Therefore, it is conceivable that hormonally mediated alterations of PEPCK-C gene expression ultimately regulate glyceroneogenesis in both liver and adipose tissue.

Lipid is released from the adipose tissue as free fatty acids and from the liver as triglycerides. Thus, glyceroneogenesis affects lipid metabolism in opposite ways in the two tissues; it restrains fat release from adipose tissue (57) and enhances it from the liver (58). It has been shown in rats that adrenalectomy enhances glyceroneogenesis and diminishes free fatty acid release from incubated epididymal fat pads (59, 60). In a reciprocal experiment, the addition of dexamethasone to cultured hepatocytes stimulated the synthesis of triglycerides and apolipoproteins E and B, as well as stimulating the release of very low density lipoproteins to the medium (58). How then is lipid homeostasis coordinated between the two tissues? We propose that the reciprocal regulation of PEPCK-C gene transcription by glucocorticoids provides a mechanism for such coordination because it represses PEPCK-C gene transcription in the adipose tissue and simultaneously enhances it in the liver. Experiments to further test this hypothesis in vivo are under way.

Acknowledgments—We are grateful to Dr. Keith Yamamoto for the wild type and mutant rat GR, to Dr. Bruce Spiegelman for supplying the PPARγ expression vector, to Dr. Daniel Lane for the C/EBPα expression vector, and to Dr. Ron Evans for the human GR and mouse RXRα expression vectors. We especially appreciate the gift from Dr. Steven L. McKnight of the anti-C/EBPα antiserum, the valuable advice during the transfection experiments in confluent NIH3T3 given by Dr. Peter Tontonoz, and the many fruitful discussions with Dr. Oded Meyuhas.

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