Peroxisome Proliferator-activated Receptor γ and Chicken Ovalbumin Upstream Promoter Transcription Factor II Negatively Regulate the Phosphoenolpyruvate Carboxykinase Promoter via a Common Element*

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A heterodimer of peroxisome proliferator-activated receptor γ (PPARγ) and retinoid X receptor (RXR) is required for adipocyte differentiation. The gene encoding cytosolic phosphoenolpyruvate carboxykinase (PEPCK) is a PPARγ/RXR target gene in adipose tissue. Of the two PPARγ response elements, gAF1/PCK1 and PCK2, only PCK2 is required for PEPCK expression and responsiveness to the PPARγ agonist, rosiglitazone, in adipose tissue even though both elements bind PPARγ/RXR in vitro. In contrast, gAF1/PCK1 is essential for glucocorticoid inhibition of PPARγ-induced PEPCK gene expression in adipocytes. We report that chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) is the predominant nuclear receptor bound to gAF1/PCK1 in preadipocytes. COUP-TFII declines during adipogenesis in reciprocal fashion to PPARγ. In transiently transfected fibroblasts COUP-TFII acts at gAF1/PCK1 to inhibit PPARγ/RXR activation via PCK2. In contrast COUP-TFII are transcriptional activators of PEPCK in hepatocytes. PPARγ/RXR occupies gAF1/PCK1 in adipocytes, and mutation of gAF1/PCK1 enhances PEPCK promoter transcriptional activity by PPARγ/RXR in fibroblasts, suggesting that this element is also a negative PPARγ response element. These results indicate that gAF1/PCK1 is a pleiotropic element through which COUP-TFII inhibits premature PEPCK expression, and perhaps adipogenesis in general, and PPARγ/RXR uses this same element in adipocytes to participate in PEPCK modulation by glucocorticoids.

The nuclear receptor superfamily consists of over 100 known members of ligand-activated transcription factors including the receptors for steroid hormones, thyroid hormone, vitamin D, retinoids, and numerous orphan receptors (reviewed in Ref. 1). The peroxisome proliferator-activated receptor (PPAR) subfamily consists of three mammalian isoforms: PPARα, PPARβ, and PPARγ (reviewed in Ref. 2). The PPARs are receptors for a wide range of both natural and xenobiotic compounds including fatty acids, eicosanoids, hypolipidemic drugs, non-steroidal anti-inflammatory drugs, and hypoglycemic drugs (2). PPARs regulate numerous genes, especially those encoding proteins involved in lipid metabolism. All three PPARs heterodimerize with retinoid X receptors (RXRs) to bind single-spaced direct repeat (DR1) response elements (5′-AGGNC A AGGTCA-3′) to which a 5′ AACT extension appears to be important for polarity and selectivity of recognition (2).

The PPARγ isofrom has a number of unique properties. It is most abundant in adipose tissue, activated macrophages, and placenta (3–6). Gene ablation experiments in mice showed that PPARγ is required for the development of placental blood vessels and adipose tissue (6–8). In addition to these developmental roles, PPARγ promotes lipid storage during adipogenesis (2, 4) and inhibits inflammation by causing apoptosis in macrophages (9, 10). All of these functions require the binding of an activating ligand such as various fatty acids, 15-deoxy-α12,14-prostaglandin J2, and thiazolidinediones, which are the newest class of drugs for treating type 2 diabetes (11–13). Finally, recent studies have implicated PPARγ in the etiology of several diseases including atherosclerosis (14, 15), hypertension (16), diabetes (16), morbid obesity (17), and cancer (18–22). Consequently, studies of PPARγ action have implications in diabetes, cardiovascular diseases, obesity, reproduction, and cancer.

Cytosolic phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32) is expressed in several tissues where it participates in glucose and lipid homeostasis (reviewed in Refs. 23 and 24). PEPCK has long been recognized as a critical gluconeogenic enzyme in the liver and kidney. Indeed, recent homologous recombination experiments confirmed the expectation that the gluconeogenic function of PEPCK is essential for life (25).

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1 The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; PEPCK, cytosolic phosphoenolpyruvate carboxykinase (EC 4.1.1.32); RXR, retinoid X receptor; COUP-TFII, chicken ovalbumin upstream promoter transcription factor II; DR1, direct repeat of two 5′-AGGNC A AGGTCA-3′ DNA motifs separated by a single nucleotide spacer; PPRE, DR1 DNA response element for a peroxisome proliferator-activated receptor γ-retinoid X receptor heterodimer; gAF1/PCK1 and PCK2, direct repeat 1 elements centered at −457 and −993 base pairs, respectively, on the rat cytosolic phosphoenolpyruvate carboxykinase promoter (GenBank accession no. K03243); DMEM, Dulbecco’s modified Eagle’s medium; bp, base pairs; RAR, retinoic acid receptor.

2 R. W. Hanson, personal communication.
PEPCK is also expressed in adipocytes, which lack the terminal two enzymes of gluconeogenesis (23, 24, 26). Consequently, adipocyte PEPCK functions in a pathway known as glyceroneogenesis (24).

Glyceroneogenesis accounts for 10–60% of triglyceride production during fasting in humans (27). Adipocyte PEPCK and glyceroneogenesis are induced during fasting to increase the production of 3-glycerophosphate for the reesterification of free fatty acids released by lipolysis. Although there is a net increase in fatty acid release, this apparent futile cycle prevents an all-out release that would otherwise cause ketosis. An additional role of hepatic PEPCK in lipid homeostasis was recently discovered in mice with a liver-specific ablation of the PEPCK gene. These mice develop fatty livers apparently because glyceroneogenesis is an important function of PEPCK in hepatocytes (25, 27).

Because of the need to regulate both gluconeogenesis and glyceroneogenesis, PEPCK gene expression is tightly controlled at the level of transcription by a variety of hormones involved in nutrient homeostasis (24). Work in many laboratories has shown that, in general, the same effectors control PEPCK in both adipocytes and hepatocytes (24, 28). One notable difference is that PPARγ regulates adipocyte but not hepatocyte PEPCK (29–31). A second difference is that glucocorticoids repress PEPCK transcription in adipocytes but induce it in hepatocytes (24). Physiologically, this latter tissue-specific difference causes stress hormones such as cortisol to increase fuel release in both tissues by stimulating hepatocyte and inhibiting adipocyte PEPCK (24, 32, 33).

We have investigated the control of PEPCK gene expression in adipocytes and shown that its gene is a target for regulation by PPARγ/ RXR (13, 29, 30, 34). We identified two PPARα/RXR binding sites: gAF1/PCK1 located 450 bp upstream, and PCK2 located 990 bp upstream of the PEPCK gene. Both sites bind recombinant PPARα/RXR with similar affinities (29, 30), yet paradoxically, these two sites have strikingly different functions. The distal site, PCK2, is absolutely required for the expression of the PEPCK promoter in adipose tissue of transgenic mice and is thus the key element of an adipocyte-specific enhancer (29). This site is also the only response element for thiazolidinedione action on the PEPCK gene. PCK2 could also mediate fatty acid induction of PEPCK in adipocytes, although this is not yet proven (13). In contrast, the proximal site, gAF1/PCK1, is neither a thiazolidinedione response element nor does it developmentally regulate adipocyte-specific PEPCK promoter expression in transgenic mice (29). However, other studies suggest that gAF1/PCK1 plays an important role in regulating PEPCK gene expression in fully differentiated cells.

First, the gAF1/PCK1 element is part of a complex hormone response unit in hepatoma cells (37, 38). It is named gAF1 (glucocorticoid-Accessory Factor 1 site) because it is required for full glucocorticoid responsiveness (37, 38). It is also called PCK1 to reflect its similarity to PCK2 (30). In hepatocytes, gAF1/PCK1 is bound by a variety of nuclear receptors including COUP-TFs, HNF4,RAR/RXR, and PPARα that activate the PEPCK promoter (39–41). Second, glucocorticoids inhibit fluoride and thiazolidinedione-induced PEPCK gene expression in adipocytes (31), but when gAF1/PCK1 is mutated, glucocorticoids no longer inhibit fluoride-induced PEPCK reporter activation (42). Thus, gAF1/PCK1 is an inhibitory element in adipocytes. Because gAF1/PCK1 binds multiple nuclear receptors and functions in glucocorticoid and retinoic acid signaling in the liver and adipose tissues, we postulated that gAF1/PCK1 regulates adipocyte PEPCK transcription by binding nuclear receptors other than PPARγ in vivo.

In this study, we compared the functions of the two PPARγ binding elements. We report that COUP-TFII binds gAF1/PCK1 and inhibits PPARγ/RXR activation via PCK2 in fibroblasts and preadipocytes. During adipogenesis PPARγ/RXR replaces COUP-TFII on gAF1/PCK1. Moreover, an intact gAF1/PCK1 element inhibits maximal PPARγ/RXR activation of the PEPCK promoter in transfection assays. We suggest that gAF1/PCK1 is a multifunctional element that mediates tissue-specific inhibition of PEPCK transcription.

**EXPERIMENTAL PROCEDURES**

**Materials—** Dulbecco’s modified Eagle’s medium (DMEM) was from Life Technologies, Inc. or Fisher. Newborn and fetal calf sera were from Sigma-Aldrich or HyClone Laboratories (Logan, UT). Oligonucleotides were synthesized by Midland Certified Reagent Co. (Midland, TX). Restriction and modifying enzymes were obtained from either PerkinElmer Life Sciences or Amersham Pharmacia Biotech. Hydroxyl-n blotting membranes and Hyperfilm were from Amersham Pharmacia Biotech. The random primer labeling kits were from Stratagene (La Jolla, CA); a pan-specific anti-COUP-TF was from Ming-Jer Tsai (Baylor College of Medicine, Houston, TX) (43); the anti-PPARγ was from Bruce Spiegelman (Dana Farber Cancer Institute, Boston, MA) (44); and anti-RXRα was from Ron Evans as described previously (Salk Institute, San Diego, CA) (30). Plasmids used to produce recombinant proteins were from the following sources: RXRαSPORT was from Ron Evans, PPARγSPORT was from Bruce Spiegelman, and the COUP-TFII and COUP-TFII expression vectors were from Ming-Jer Tsai. Rosiglitazone (BRL49653) was a gift from GlaxoSmithKline (Middlesex, United Kingdom). All other chemicals were analytical grade from Fisher or Sigma-Aldrich.

**RNA Extraction and Analysis—** RNA was extracted by the method of Chomczynski and Sacchi (45). Total RNA (20 μg) was electrophoresed on a 1% agarose gel containing 2.2% formaldehyde and transferred to Hybond-N membranes. The integrity and relative amounts of RNA were assayed by methylene blue staining. Membranes were prehybridized for 30 min at 65°C in 0.25 M sodium phosphate (pH 6.8), 1 × EDTA containing 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin. Hybridization was performed overnight at 65°C with 10 000 to 100 000 cpm/ml of cDNA labeled with [32P]dATP by random priming, synthesized according to the manufacturer’s recommendations. Membranes were washed twice for 15 min at room temperature in 2× SSC (1× SSC in 0.3 M NaCl, 0.03 M sodium citrate, pH 7.1, 0.1% SDS) and then for 30 min at 60°C with 0.1× SSC, 0.1% SDS. The PEPCK cDNA probe was PC116, a rat PEPCK cDNA fragment (46). The murine PPARγ cDNA probe (nucleotides 25–879) was obtained by EcoRI digestion of pSGS-PPARγ (gift from E. Z. Amori and P. Grimaldi). The COUP-TFII cDNA probe (nucleotides 1–870) was obtained by EcoRI and PstI digestion of pMT2-Arp1 (gift from S. Karathanasis). The mRNA signal was quantified by scanning densitometry and was corrected for differences in RNA loading by comparing the signals generated by either the β-actin cDNA probe (47) or an 18 S rRNA oligonucleotide probe.

**Cell Culture and Transfections—** 3T3-L1 fibroblasts were obtained from ATCC (catalog no. CL-173), differentiated into adipocytes, and cultured as described previously (29). 3T3-F442A cells (able to differentiate into adipocytes) and 3T3-C2 (control cells unable to differentiate into adipocytes) (48) were cultured as described previously (49, 50). NIH-3T3 cells were cultured following the protocol of Frost and Lane (51) in DMEM containing 10% calf serum that had been stripped with activated charcoal and Bio-Rad AG1-X8 anion exchange resin (52). NIH-3T3 cells were transfected by calcium phosphate precipitation as described previously (29).

A Renilla luciferase vector, pRL-CMV, from Promega (Madison, WI), was included in all experiments as a transfection efficiency control. Firefly and Renilla luciferase activities were assayed using the dual luciferase reporter assay kit (Promega). Luminescence was measured with an Analytical Luminescence Laboratories Monolight 2010 luminometer (PharMingen, San Diego, CA).

**Statistics—** A least squares analysis of variance was performed using SAS on a logarithmic transformation of the transcription data. The factors used in the statistical model are listed in each figure legend. Significance was determined with the predicted difference test in the General Linear Models procedure of the SAS program.
The proteins bound to gAF1/PCK1 and PCK2 are similar during adipocyte differentiation. A, rat gAF1/PCK1 and PCK2 are compared with a consensus PPRE. Shaded bases are conserved with the consensus sequence (35, 36). Arrows above the sequences represent the direct repeats. B, electrophoretic mobility shift assays were performed using 4 pmol of a 32P-end-labeled oligonucleotide probe containing either double-stranded gAF1/PCK1 or PCK2. Recombinant PPARγ and RXRα (RX/Rα) were synthesized in a coupled in vitro transcription, translation system and used as a control (indicated by arrow A). Nuclear extracts were prepared from 3T3-L1 cells at the indicated times of adipocyte differentiation. DNA-protein complexes were electrophoresed on a 5% polyacrylamide gel and exposed to a PhosphorImager (Molecular Dynamics) as described under “Experimental Procedures.”

Nuclear Extracts and in Vitro Translated Proteins—In vitro transcription/translation of RXRα-SPORT and PPARγ-SPORT plasmids was performed with the TNT SP6-coupled reticulocyte lysate system, whereas pBSK-COUP-TFI and pBSK-COUP-TFII used the T7 system as recommended by the manufacturer (Promega). Two to three μl of the 50-μl translation product was used in each binding reaction. Final protein concentrations were typically 1–2 μg/ml as determined by BCA protein assays (Pierce). 3T3-L1 preadipocyte and adipocyte nuclear extracts were prepared as described previously (29). Nuclear proteins were extracted from 3T3-F442A preadipocytes and adipocytes using the method described by Shapiro et al. (53).

Electrophoretic Mobility Shift Assays—DNA-protein binding was performed as described previously (29, 42). Briefly, rat PEPCK double-stranded oligonucleotide probes were end labeled by filling the 3′- recessed ends with [α-32P]dATP or [-γ-32P]dGTP (3000 Ci/mmol) using the large fragment of Escherichia coli DNA polymerase I (Klenow fragment). The −467 to −352 PEPCK probe was generated by including 60 μCi of [α-32P]dATP (6000 Ci/mmol) into a 10-μl PCR reaction using a subclone of the rat PEPCK gene (GenBank® accession no. K03243) as template. The 32P-labeled PCR product was purified with Qiagen II, as recommended by the manufacturer (Qiagen, Valencia, CA). The PCR primers were: 5′-CTGAAATTCCCTTCTCATCAGC-3′ (sense) and 5′-GGAGACTTCCATATGCTGCTG-3′ (antisense). DNA-protein complexes were resolved on 5% polyacrylamide gel (29:1 acrylamide:bisacrylamide) in 1× TBE (50 mM Tris borate (pH 8.3), 1 mM EDTA). The gel was dried and exposed to a phosphor screen overnight. When autoradiogram was used, binding reactions were incubated with 1 μl of antiserum for 15 min at room temperature prior to the addition of the labeled fragments.

Reporters and Mutagenesis—PEPCK promoter-luciferase vectors were constructed using the pGL3-basic firefly luciferase vector from Promega. The wild type PEPCK-luciferase vector utilized the same BamHI (~1300 bp)/BglII (~600 bp) rat PEPCK promoter fragment described previously (29, 30). This fragment was ligated into pGL3-basic, which had been digested with SmaI and BglII. Mutations in gAF1/PCK1 and PCK2 were generated by site-directed mutagenesis with a QuikChange® site-directed mutagenesis kit from Stratagene using a thermocycler (ERICOMP, San Diego, CA). Oligonucleotide sequences are as follows (mutated bases are in bold/underlined text): PCK2M(−): 5′-CCCTTCTCATGACGATCGCGCTGGGAGTCACACCC-3′; PCK1M(−): 5′-CCCTTCTCATGACGCCGCTGGGAGTCACACCC-3′. The introduction of a BamHI (PCK2M) or an EagI restriction site (PCK1M) allowed quick confirmation of the mutations. Upon positive screening, appropriate restriction enzymes were used to cleave a fragment containing the mutation, which was then ligated back into the original parent vector. The mutations were confirmed by automated sequencing in the Texas Tech University Biotechnology Core Facility.

RESULTS

Two DR1 elements upstream of the PEPCK gene, gAF1/PCK1 and PCK2, bind PPARγ/RXR in vitro (30, 35). However, transgenic mouse experiments revealed a distinct functional difference between the two sites, since only PCK2 is required for adipocyte-specific PEPCK expression (29). Oligonucleotides spanning gAF1/PCK1 and PCK2 (Fig. 1A) were assessed for binding differences using electrophoretic mobility shift assays with nuclear extracts from 3T3-L1 cells at various stages of differentiation. The binding patterns to both gAF1/PCK1 and PCK2 were very similar throughout the entire differentiation period except in preadipocytes (Fig. 1B). We observed subtle differences in the protein species bound to gAF1/PCK1 compared with PCK2 in preadipocytes (Fig. 1B, compare lanes 2 and 11; see Ref. 29). Together, the available evidence indicated that the major change that occurs throughout adipogenesis is the appearance of PPARγ. It thus seemed that PPARγ/RXR binds equally to gAF1/PCK1 and PCK2, but, for unknown reasons, the complex at gAF1/PCK1 does not effectively trans-activate the PEPCK promoter.

PPARγ appears early in adipogenesis, 2–3 days before PEPCK gene expression is detectable (30). This could result from 1) an inactive or missing stimulatory factor(s) in preadipocytes, or 2) an inhibitory factor that is present in preadipocytes and disappears during adipogenesis. The experiments shown in Fig. 2 address the first possibility by determining
whether existing regulatory pathways could be activated. An electrophoretic mobility shift assay, using a radiolabeled PCK2 double-stranded oligonucleotide as probe, was used to assess PPAR expression before and after differentiation (Fig. 2A). Using specific antisera, we compared the binding of PPARγ or PPARα to PCK2 in nuclear extracts isolated from confluent 3T3-F442A preadipocytes or adipocytes. PPARα was undetectable in both cell types, whereas PPARγ (presumably as a heterodimer with a constitutively present RXR) was the primary species bound to PCK2 in adipocyte nuclear extracts. PPARγ was also a significant binding species in confluent preadipocytes albeit at lower levels than in adipocytes. We next tested whether cAMP, all-trans-retinoic acid, 9-cis-retinoic acid, and the thiazolidinedione, rosiglitazone could induce PCK2 mRNA in confluent preadipocytes. PEPCCK mRNA was not expressed in preadipocytes, irrespective of the addition of activators for 4 h (Fig. 2B) or 24 h (data not shown). In contrast, PEPCCK mRNA was induced as expected in adipocytes by all four effectors (Fig. 2C). Clearly, PEPCCK mRNA is refractory to induction under these conditions in preadipocytes, which is consistent with the possible presence of an inhibitor prior to terminal differentiation.

The possibility that gAF1/PCK1 mediates inhibitory responses is supported by the observation that this PPARγ/RXR-binding element is required for glucocorticoids to inhibit thiazolidinedione-induced PEPCCK gene expression in adipocytes (42). We postulated that COUP-TFI and COUP-TFII bind PPAR response elements and block PPARγ action (54–58). Second, COUP-TFs are well documented regulators of hepatic PEPCCK expression via gAF1/PCK1 (39–41). Third, COUP-TFs are in preadipocytes and inhibit adipogenesis (56). It may also be significant that there was a substantial amount of residual PCK2-binding species that was not affected by the presence of PPARγ antibody (Fig. 2A). It was thus possible that an inhibitory nuclear receptor might also bind to gAF1/PCK1.

We expected that factors other than PPARγ/RXR would occupy gAF1/PCK1 in adipocytes since this element does not function like PCK2 (29). We therefore used mobility shift assays with antibodies to PPARγ, RXRα, and COUP-TF to determine which nuclear receptors bind to gAF1/PCK1 in nuclear extracts from preadipocytes and adipocytes (Fig. 3). The COUP-TF antibody used in this experiment recognizes both COUP-TFI and COUP-TFII (43). COUP-TF antisera retarded (lane 2, arrow A) or eliminated most of the binding activity in the preadipocyte extracts, whereas there was no change in adipocytes (lane 6). The PPARγ antibody caused a slight diminution of binding (compare lanes 1 and 3), and a supershifted band was seen in the presence of the RXRα antisense in preadipocytes (lane 4, arrow B). In contrast, the gAF1/PCK1 occupants differ in terminally differentiated adipocytes since anti-COUP-TF had little effect on the mobility shift patterns (compare lanes 5 and 6), whereas PPARγ and RXRα antibodies eliminated or supershifted most of the binding species (arrow B, lanes 7 and 8). These results indicate that COUP-TF is the major occupant of gAF1/PCK1 in preadipocytes, although a small amount of PPARγ/RXR also binds. Thus, during differentiation there was a shift in the factors bound to gAF1/PCK1 from COUP-TF in preadipocytes to PPARγ/RXRα in adipocytes.

We next asked whether the gAF1/PCK1-binding species was COUP-TFI or COUP-TFII. Fig. 4 shows an experiment using isotype-specific COUP-TF antibodies to supershift recombinant COUP-TFI and COUP-TFII that had been synthesized in a coupled transcription-translation system. This experiment shows that both COUP-TFI and COUP-TFII are capable of binding to gAF1/PCK1 and PCK2 since recombinant COUP-TF generates a unique band (arrow B) that is supershifted by the respective antibody (arrow A). These isotype-specific antibodies were next used to examine which COUP-TF species binds gAF1/PCK1 at various times throughout adipogenesis (Fig. 5). In confluent preadipocytes, both PPARγ (arrow A, lane 2) and COUP-TFII (arrow A, lane 4) were among the occupants of gAF1/PCK1. No COUP-TFI was detectable. We noted that the anti-COUP-TFII antibody did not affect a substantial amount of gAF1/PCK1-binding activity (bracket B, lane 5). This is in contrast to the pan-specific COUP-TF antibody used in Fig. 3 that blocked or supershifted most of the species that bound.
The observation that not all of the recombinant COUP-TFII was supershifted in Fig. 4 indicates that the antibody was limiting. However, doubling the amount of antibody did not increase the relative amount of radioactivity in the supershifted band (data not shown). Thus, it is not known whether the residual species (B in Fig. 5A) represent unbound COUP-TFII or additional nuclear receptor species. COUP-TFII then disappeared and was replaced by PPARγ/H9253 on day 3 of differentiation (Fig. 5A, lanes 6, 8, and 10), although a small amount of COUP-TFII seemed to be present on day 12 (lane 12).

The occupancy of gAF1/PCK1 by PPARγ in adipocytes was an apparent paradox in that it would seem that this element should behave as an adipocyte-specific enhancer like PCK2 (29). However, our previous data demonstrated that gAF1/PCK1 is not an enhancer, but it mediates the negative effects of glucocorticoids in adipocytes (29, 42). It seemed unlikely that PPARγ/RXR would serve as both activator and inhibitor via separate elements on the same gene in the same cell type. We therefore postulated that a factor other than PPARγ binds when gAF1/PCK1 is in the context of its normal flanking DNA.

To test this possibility, an electrophoretic mobility shift assay was performed using the region from −467 to −352 bp of the PEPCK promoter (B) as probes. 3T3-L1 nuclear extracts isolated at the indicated times of adipogenesis were tested for binding. Brackets indicate the major retarded complexes (B). Species supershifted by the indicated antibodies are indicated by an arrow (A). In panel B, the DNA protein complexes corresponding to factors bound to gAF1/PCK1 complex were identified by competition with 100-fold molar excess of unlabeled gAF1/PCK1 (lanes 4 and 12).

**Fig. 4.** Recombinant COUP-TF binds to both PEPCK DR1 elements. Electrophoretic mobility shift assays were performed using recombinant COUP-TFI and COUP-TFII proteins synthesized in a coupled in vitro transcription/translation system. Specific COUP-TF binding is indicated by arrow B. To demonstrate protein binding specificity, antisera to COUP-TFI (TFI) or COUP-TFII (TFII) were added to the reaction and used to supershift the COUP-TF-DNA complexes (arrow A).

**Fig. 5.** COUP-TFII binds to gAF1/PCK1 in preadipocytes. Electrophoretic mobility shift assays were performed as described under “Experimental Procedures” using gAF1/PCK1 alone (A) or gAF1/PCK1 with its flanking DNA in a 116-bp region extending from −467 to −352 bp of the PEPCK promoter (B) as probes. 3T3-L1 nuclear extracts isolated at the indicated times of adipogenesis were tested for binding. Brackets indicate the major retarded complexes (B). Species supershifted by the indicated antibodies are indicated by an arrow (A). In panel B, the DNA protein complexes corresponding to factors bound to gAF1/PCK1 complex were identified by competition with 100-fold molar excess of unlabeled gAF1/PCK1 (lanes 4 and 12).
FIG. 6. COUP-TFII and PPARγ mRNA levels change in 3T3-F442A but not 3T3-C2 cells. Total RNA (20 μg/lane) was resolved by electrophoresis, blotted to nylon, and hybridized with 32P-labeled mouse PPARγ2 (panel A) or human COUP-TFII (panels B and C) cDNA probes as described under “Experimental Procedures.” The data were normalized for equivalent amounts of intact RNA by hybridization to an 18S rRNA oligomer probe. Data in panels A and B are expressed as the percentage of the mRNA signal in confluent cells (day 0). Each value shown in panel B represents the mean ± S.E. of results obtained from three independent experiments including the representative Northern blot shown in panel C.

lanes 6 and 9). COUP-TFII antiserum eliminated most of the binding activity in preadipocytes, had no effect at day 3 of differentiation, and blocked a portion of the binding at day 12 (lanes 3, 7, 10, and 12). Thus, context did not substantially affect the factors bound to gAF1/PCK1 throughout adipogenesis.

To confirm that COUP-TFII but not COUP-TFI was present in preadipocytes and decreases while PPARγ increases during differentiation, total RNA was isolated from 3T3-F442A cells at various times during adipogenesis and analyzed by Northern blotting (Fig. 6). The closely related non-adipogenic 3T3-C2 fibroblast cell line was used as a negative control. As expected, PPARγ mRNA increased as differentiation proceeded in 3T3-F442A cells, but not in 3T3-C2 cells (Fig. 6A). COUP-TFII mRNA decreased ~4-fold during 3T3-F442A differentiation but not in 3T3-C2 cells under the same conditions (Fig. 6B and C). Little if any COUP-TFI mRNA was detected in either of these cell types (data not shown).

We next asked whether COUP-TFII negatively regulates expression of the PEPCK promoter in fibroblasts. This question was first addressed using transient transfection assays in NIH-3T3 cells cotransfected with a wild type PEPCK-luciferase reporter construct plus expression vectors encoding PPARγ, RXRα, and COUP-TFII (Fig. 7). COUP-TFII alone did not affect PEPCK reporter activity, whereas PPARγ/RXRα caused a large transactivation (5-fold in this experiment). However, COUP-TFII inhibited PPARγ/RXRα transactivation in a dose-dependent fashion. We next asked whether this COUP-TFII-mediated transcriptional inhibition requires gAF1/PCK1. Transient transfection assays were performed as in Fig. 7 but with PEPCK reporter constructs containing either the wild type or a gAF1/PCK1 mutation (gAF1/PCK1M). As in Fig. 7, COUP-TFII repressed PPARγ/RXRα transactivation of the wild-type PEPCK reporter gene by about 50% (Fig. 8). However, COUP-TFII did not repress PPARγ/RXRα transactivation of the gAF1/PCK1M reporter. Thus, an intact gAF1/PCK1 element was required for COUP-TFII inhibition of PPARγ-induced PEPCK gene expression, suggesting that gAF1/PCK1 is the target element for COUP-TFII in preadipocytes. This also eliminates the possibility that the mechanism of inhibition results from binding of essential factors to COUP-TFII in solution.

Finally, we asked whether PPARγ/RXR could also negatively regulate expression of the PEPCK promoter via gAF1/PCK1 in fibroblasts. To test this possibility, we conducted transient transfection assays in NIH-3T3 fibroblasts (Fig. 9). Each of three PEPCK-luciferase reporter constructs (wild type; a gAF1/PCK1 mutant, PCK1M; and a PCK2 mutant, PCK2M) were tested for transactivation by PPARγ/RXR (Fig. 9). Co-transfection of the wild type construct along with 188 ng of each of PPARγ and RXRα expression vectors resulted in a 25-fold increase in luciferase activity in the presence of 1 μM rosiglitazone, a PPARγ activator. Activation of the wild type construct was maximal with 188 ng because co-transfection with 375 or 750 ng of PPARγ and RXRα expression vectors resulted in essentially the same level of luciferase activity. As expected from earlier studies (29, 30), mutation of PCK2 abolished all transactivation by PPARγ/RXRα. In contrast, there was a PPARγ/
Fig. 8. gAF1/PCK1 is required for COUP-TFII-dependent inhibition. Transient transactivation assays were performed in NIH-3T3 cells as described in Fig. 7 using both the wild type (open bars) and gAF1/PCK1M (closed bars) PEPCK reporters. PPARγ/RXRα (375 ng each) and/or COUP-TFII (750 ng) were co-transfected where indicated. Luciferase values are normalized relative to the activation of the wild type reporter by PPARγ/RXRα (set to 100). Data represent the means ± S.E. of three independent experiments. Statistics were performed as described under “Experimental Procedures” using SAS. COUP-TFII concentration, PPARγ/RXR presence, and reporter mutation were the factors included in the statistical model. *, not different from each other, but different from the activity of untransfected cells, p < 0.0001; #, p < 0.0001 when activity is compared with the activity of cells transfected only with PPARγ/RXRα.

Fig. 9. gAF1/PCK1 in a negative PPRE. Transient transactivation assays were performed in NIH-3T3 cells as described in Fig. 7 using the wild type (open bars), PCK2 mutant (PCK2M, black bars), and gAF1/PCK1 mutant (gAF1/PCK1M, gray bars) PEPCK-luciferase reporters illustrated above the graph. The data were normalized for transfection efficiency using 0.05 μg of CMV-Renilla luciferase control reporter, and luciferase values were normalized relative to the activation by 188 ng of PPARγ/RXRα on the wild type reporter (set to 100). Data represent the means ± S.E. of three independent experiments. Statistics were performed as described under “Experimental Procedures” using SAS. Reporter mutation and PPARγ/RXR concentration were the factors included in the statistical model. *, not different from each other, but different from reporter alone (p < 0.01); †, not different from each other; #, different from each other (p < 0.05) and all different from reporter alone (p < 0.05).

RXRα concentration-dependent increase in luciferase activity with the gAF1/PCK1 mutant reporter. More importantly, the maximal response of the gAF1/PCK1 mutant was significantly higher than that of the wild type promoter. These data suggest that gAF1/PCK1 can serve as a negative PPRE in fibroblasts.

**DISCUSSION**

The evidence presented in this study indicates that gAF1/PCK1 mediates inhibition of the PEPCK promoter by COUP-TFII in preadipocytes and by PPARγ/RXR and possibly other factors in adipocytes. Briefly, the evidence is as follows. The inability to induce the expression of PEPCK in preadipocytes that express PPARγ suggests the presence of an inhibitor (Fig. 2). COUP-TFII occupies gAF1/PCK1 in fibroblasts prior to differentiation (Figs. 3–6), and inhibits PEPCK promoter transactivation via gAF1/PCK1 in transiently transfected fibroblasts (Figs. 7 and 8). PPARγ/RXR becomes a gAF1/PCK1 occupant in terminally differentiated adipocytes (Figs. 3 and 5), and transactivation of the PEPCK promoter is enhanced in transiently transfected fibroblasts if gAF1/PCK1 is mutated (Fig. 9).

These experiments reveal a new tissue-specific role for gAF1/PCK1. In preadipocytes and fibroblasts, gAF1/PCK1 mediates inhibition of PEPCK promoter expression. It also mediates glucocorticoid inhibition of PPARγ-induced PEPCK expression in adipocytes (42). In contrast, RAR/RAR, RAR/thyroid receptor, RAR/RXR, COUP-TF, and HNF-4 activate expression of the PEPCK gene via gAF1/PCK1 in hepatocytes (39, 40, 59, 60). Thus, gAF1/PCK1 is a tissue-specific pleiotropic element in that it binds a variety of nuclear receptors, and it is inhibitory in some cell types and stimulatory in others.

It is likely that factors in addition to COUP-TFII and PPARγ/RXR bind gAF1/PCK1 in preadipocytes and adipocytes. The basis for this assertion is that there was always some residual binding species that remained unaffected by the addition of specific antibodies in supershift experiments (see Figs. 2, 3, and 5 and Footnote 3). Addition of more antibody did not decrease the residual binding species suggesting that antibody was not limiting. The amount of residual binding activity varied among experiments (data not shown), suggesting varying proportions of the gAF1/PCK1-binding species. We speculate that these additional factors compete with PPARγ and COUP-TFII. Their identities and whether they are inhibitory remains to be determined.

This study also revealed a striking functional difference between the two DR1 elements upstream of the PEPCK promoter. The distal DR1, PCK2, is a strong positive PPRE that is required for adipocyte-specific expression of the PEPCK promoter and is a thiazolidinedione response element (29). In contrast, the proximal DR1, gAF1/PCK1, is an inhibitory PPRE in fibroblasts (Fig. 9) and adipocytes (42). This raises an interesting mechanistic question of how PPARγ/RXR can have both a positive and a negative action on the PEPCK promoter in a single cell type.

Fig. 10 illustrates a model of the pleiotropic tissue-specific roles of gAF1/PCK1. It integrates published work (especially Refs. 42 and 59) with the results reported here and elsewhere. We propose that COUP-TFII occupies gAF1/PCK1 in preadipocytes and blocks activation of the PEPCK promoter via PPARγ/RXR bound to PCK2. This could represent a general mechanism to block premature adipogenesis until a critical PPARγ-COUP-TFII ratio is reached. Indeed, COUP-TFII is known to inhibit the expression of several PPARγ-regulated genes in adipocytes (54–57, 61).

We propose that gAF1/PCK1 takes on a new role in mature adipocytes where it mediates glucocorticoid repression of PEPCK expression by blocking the action of PPARγ/RXR bound to PCK2. According to this model, binding of glucocorticoids to their receptors signals the factors bound to gAF1/PCK1 to
inhibit PPARγ/RXR action from PCK2. The gAF1/PCK1-binding factors include PPARγ/RXR and other unidentified proteins. Two observations suggest that a positive signal from the glucocorticoid receptors is required to activate the inhibitory pathway from gAF1/PCK1 in adipocytes. The first is the loss of the glucocorticoid effect when gAF1/PCK1 is mutated (42). The second is the lack of an effect of a gAF1/PCK1 mutation on expression of a PEPCK-CAT reporter construct in transient transfection assays in mature adipocytes. We cannot yet explain why input from a glucocorticoid response pathway is not needed to inhibit PEPCK expression in fibroblasts and preadipocytes.

The final part of the model in Fig. 10 illustrates the function of gAF1/PCK1 in the liver. In hepatocytes, gAF1/PCK1 is a critical component of a complex control domain (the glucocorticoid response unit) that mediates responsiveness to glucocorticoids but inhibit it in adipocytes via gAF1/PCK1 may have a common physiologic function in terms of energy balance during fasting and stress when these steroids are elevated. Glucocorticoids would enhance fuel production in the liver by increasing PEPCK and thus stimulating gluconeogenesis. Glucocorticoids would enhance fuel release from adipose tissue by lowering PEPCK, which would decrease gluconeogenesis and thus increase free fatty acid release into the blood. It thus seems quite logical that glucocorticoids should inhibit PPARγ action because the increased serum free fatty acids (from lipolysis) would otherwise stimulate gluconeogenesis via PPARγ and the PCK1 gene, which would oppose fatty acid mobilization.

In summary, this study brings to light two new phenomena. First, PPARγ/RXR can serve as either an activator or a repressor of the PEPCK promoter depending upon whether it is bound to PCK2 or gAF1/PCK1. The leptin gene is the only previously reported example in which PPARγ/RXR down-regulates expression of a promoter (52). However, the effect on leptin appears to be indirect, being mediated through C/EBP family members. The PEPCK promoter may thus be the first example of direct inhibition by PPARγ/RXR. Second, COUP-TFs act via gAF1/PCK1 to activate the PEPCK promoter in hepatocytes and repress it in adipocytes. These observations raise new questions that must be answered. Specifically, what causes gAF1/PCK1 to be a positive COUP-TFI1-response element in hepatocytes, but a negative element in adipocytes? In addition, what causes PPARγ/RXR to be an activator when bound to PCK2 and an inhibitor when bound to gAF1/PCK1 in some cell types? Our current working hypothesis is that DR1 context determines its function. The context of gAF1/PCK1 is almost certainly different in adipocytes and hepatocytes as a consequence of different tissue-specific factors that bind the flanking DNAs. Similarly, the contexts of gAF1/PCK1 and PCK2 differ in adipocytes because the flanking DNAs are different, and so must be the neighborhood of binding proteins. In support of this, Williams et al. (49) reported a DNase-hypersensitive site near gAF1/PCK1 in adipocytes that is missing in hepatocytes. Moreover, PCK2 hypersensitivity changes during adipogenesis, whereas gAF1/PCK1 hypersensitivity is the same in preadipocytes and adipocytes. Studies are currently under way to determine if DNA context and/or site differences mediate PPARγ action. Future studies will also need to address the physiological roles of these phenomena.

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REFERENCES
1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 835–839
2. Desvergne, B., and Wahli, W. (1999) Endocr. Rev. 20, 469–688
3. Spiegelman, B. M. (1998) Diabetes 47, 507–514
4. Spiegelman, B. M., and Flier, J. S. (1996) Cell 87, 377–389
5. Wendeling, O., Chambon, P., and Mark, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 547–551
6. Rosen, E. D., Sarraf, P., Troy, A. E., Bradwin, G., Moore, K., Milestone, D. S., Spiegelman, B. M., and Mortensen, R. M. (1999) Mol. Cell 4, 611–617
7. Kubota, N., Terauchi, Y., Miik, H., Tatemoto, H., Yamauchi, T., Kameda, K., Satoh, S., Nakano, R., Ishii, C., Sugiyama, T., Eto, K., Tsuabomo, Y., Okuno, A., Murakami, K., Sekihara, H., Hasegawa, G., Naito, M., Toyoshima, Y., Tanaka, S., Shiota, K., Kitamura, T., Fujita, T., Ezeki, O., Aizawa, S., Kadowaki, T., et al. (1999) Mol. Cell 4, 597–609
8. Barak, Y., Nelson, M. C., Ong, E. S., Jones, Y. Z., Ruiz-Lozano, P., Chien, K. R., Koder, A., and Evans, R. M. (1999) Mol. Cell 4, 585–586
9. Spiegelman, B. M. (1999) Cell 98, 153–155
10. Cinetti, G., Grigio, S., Antonucci, M., Torra, I. P., Delerive, P., Majd, Z., Fruchart, J. C., Chapman, J., Najib, J., and Staels, B. (1999) J. Biol. Chem. 274, 25573–25580
11. Kiefer, S. A., Lenhard, J. M., Wilsson, T. M., Patel, I., Morris, D. C., and Lehmann, J. M. (1995) Cell 83, 813–819
12. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) Mol. Cell Biol. 15, 241–252
13. Duplus, E., Galian, M., and Forest, C. (2000) J. Biol. Chem. 275, 30749–30752
14. Tontonoz, P., Nagy, L., Alvarez, J. G., Thomazy, V. A., and Evans, R. M. (1998) Cell 93, 241–252
15. Nagy, L., Tontonoz, P., Alvarez, J. G., Chen, H., and Evans, R. M. (1998) Cell 95, 229–240
16. Barros, J., Gurnell, M., Crowley, V. E., Agostini, M., Schwabe, J. W., Soos, M. A., Maslen, G. L., Williams, T. D., Lewis, H., Schafer, A. J., Chatterjee, A. K., Kadowaki, T., Aizawa, S., Koder, A., and Evans, R. M. (1999) Mol. Cell 3, 597–606
17. Ristow, M., Muller-Wieland, D., Pfeiffer, A., Krone, W., and Kahn, C. R. (1998) J. Biol. Chem. 273, 30749–30752
18. Tontonoz, P., Singer, S., Forman, B. M., Sarraf, P., Fletcher, J. A., Fletcher, C. D., Brun, R. P., Mueller, E., Ahitov, S., Oppenheim, H., Evans, R. M., and Spiegelman, B. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 237–241
19. Tilmann, S. M., Ballard, F. J., and Hanson, R. W. (1976) in Gluconeogenesis: Its Regulation in Mammalian Species (Hanson, R. W., and Mehlman, M. A., eds) pp. 47–91, John Wiley & Sons, New York
20. Hanson, R. W., and Reshef, L. (1977) Annu. Rev. Biochem. 46, 581–611
21. She, P., Shiota, M., Shelton, K. D., Chaklery, R., Postic, C., and Magnuson, M. A. (2000) Mol. Cell. Biol. 20, 6508–6517
