Transcription Coactivator PRIP, the Peroxisome Proliferator-Activated Receptor (PPAR)-Interacting Protein, Is Redundant for the Function of Nuclear Receptors PPARα and CAR, the Constitutive Androstane Receptor, in Mouse Liver

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Disruption of the genes encoding for the transcription coactivators, peroxisome proliferator-activated receptor (PPAR)-interacting protein (PRIP/ASC-2/RAP250/ TRBP/NRC) and PPAR-binding protein (PBP/TRAP220/ DRIP205/MED1), results in embryonic lethality by affecting placental and multiorgan development. Targeted deletion of coactivator PBP gene in liver parenchymal cells (PBP$^{Liv-/-}$) results in the near abrogation of the induction of PPARα and CAR (constitutive androstane receptor)-regulated genes in liver. Here, we show that targeted deletion of coactivator PRIP gene in liver (PRIP$^{Liv-/-}$) does not affect the induction of PPARα-regulated pleiotropic responses, including hepatomegaly, hepatic peroxisome proliferation, and induction of mRNAs of genes involved in fatty acid oxidation system, indicating that PRIP is not essential for PPARα-mediated transcriptional activity. We also provide additional data to show that liver-specific deletion of PRIP gene does not interfere with the induction of genes regulated by nuclear receptor CAR. Furthermore, disruption of PRIP gene in liver did not alter zoxazolamine-induced paralysis, and acetaminophen-induced hepatotoxicity. Studies with adenovirally driven EGFP-CAR expression in liver demonstrated that, unlike PBP, the absence of PRIP does not prevent phenobarbital-mediated nuclear translocation/retention of the receptor CAR in liver in vivo and cultured hepatocytes in vitro. These results show that PRIP deficiency in liver does not interfere with the function of nuclear receptors PPARα and CAR. The dependence of PPARα- and CAR-regulated gene transcription on coactivator PBP but not on PRIP attests to the existence of coactivator selectivity in nuclear receptor function.

Key words: Nuclear receptor coactivators; PRIP; PBP/TRAP220/MED1; CAR; PPARα

INTRODUCTION

Nuclear receptor coactivators enhance the transcriptional activation of nuclear receptor-regulated genes and thus function as positive regulators of transcription (2,8,11,32,35,46,48). Over 100 nuclear receptor cofactors (coactivators, coactivator-associated proteins, corepressors, and others) are known to be involved in nuclear receptor-directed transcription (32,35,46), but information on the selectivity and

specificity of cofactor requirement for a given nuclear receptor in cell and gene-specific transcription is rudimentary at best (35,62). Nuclear receptors, such as constitutive androstane receptor (CAR), pregnane X receptor (PXR), steroid and xenobiotic receptor (SXR), estrogen receptor α and β, and glucocorticoid receptor belong to the type I nuclear receptor category because they are localized in the cytoplasm and require ligand activation to facilitate their translocation into the nuclear compartment (12,21,22,51). These ligand-activated type I nuclear receptors form homodimers or heterodimerize with retinoid X receptor to bind to receptor-specific sites (cognate-responsive elements) in the promoter of target genes (8,35). Nuclear receptors belonging to the type II category, such as thyroid hormone receptor (TR)-α and TRβ, and members of peroxisome proliferator-activated receptor (PPAR) subfamily, reside in the nucleus in the absence of ligand as heterodimers with retinoid X receptor (24). These heterodimerized receptors bind to DNA and are maintained in a repressed state by nuclear receptor corepressors in the absence of ligand activation (32,48). Following ligand activation, both type I and type II nuclear receptors undergo conformational changes to optimize interaction with LXXLL (L, leucine; X, any amino acid) nuclear receptor box motif(s) of transcription coactivators (8,35,48). Ligand activation of a nuclear receptor initiates an orchestrated recruitment of several transcriptional coactivators and coactivator-associated proteins that form multisubunit protein complexes to facilitate nucleosome remodeling and nuclear receptor linking to the basal transcription machinery (8,11,44,46,48,49).

Coactivators, such as p160/SRC-1 family members (SRC-1/NCoA1/p160; NCoA2/TIF2/GRIP1/SRC-2; and NCoA3/pCIP/ACTR/SRC-3), in cooperation with CBP/p300 and pCAF, form a complex with histone acetyltransferase activity (HAT complex) to remodel chromatin (7–9,19,35,39,42). Additional groups of coactivators and coactivator-associated proteins such as CARM1 and PIMT are then recruited sequentially, combinatorially, or by some other mechanism (3,35,37,52,70). Several of these proteins belong to the TRAP/DRIP/ARC/MED complex (44,48,49,63), with coactivator PBP/TRAP220/DRIP205/MED1 serving as core protein for this complex (44,63,68). Coactivator PRIP (also known as ASC-2/RAP250/TRBP/NRC) forms another large steady-state protein complex [ASC-2 complex (ASCOM)] with retinoblastoma-binding protein RBQ-3, α/β-tubulins, and trithorax group of proteins ALR-1, ALR-2, HALR, and ASH (1,9,25,30,33,67). PRIP-interacting protein PIMT binds to PRIP, CBP/p300, and some members of TRAP/MED complex to function as a linker be-

tween key proteins of HAT, TRAP, and ASCOM complexes during transcription (9,37,70).

In order to understand the in vivo biological functions of these coactivators, molecular genetic approaches are being utilized to systematically explore their role in development, metabolism, and regulation of nuclear receptor-specific gene transcription (43,46, 54,55,59,60,62). Targeted gene knockout models reveal that some of the coactivators and coactivator-associated proteins are indispensable for embryonic development while others are considered dispensable (46). Deletion of coactivator PBP, PRIP, CBP, and p300 genes is embryonic lethal, implying that these coactivators are widely involved in transcription, possibly affecting the function of many transcription factors (1,15,28,34,66,69). Conditional deletion of PBP gene (*PPARBP*) in liver parenchymal cells (PBP$^{\text{Liv}-/-}$) results in the abrogation of the effects of PPARα ligands, indicating that PBP is essential for PPARα signaling (17). Deletion of PBP gene in liver also abolishes hypertrophic and hyperplastic influences in liver mediated by CAR ligands, phenobarbital and 1,4-bis-2[-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), and of acetaminophen-induced hepatotoxicity (16). CAR ligands such as phenobarbital and TCPOBOP facilitate the translocation of CAR into the hepatocyte nucleus, and this translocation fails to occur in PBP hepatocytes, accounting for the abrogation of CAR-mediated gene expression in liver (10,12,16,21). Here we report the generation of PRIP liver conditional null mice (PRIP$^{\text{Liv}-/-}$) and present evidence to demonstrate that coactivator PRIP is not required for the functioning of nuclear receptors PPARα and CAR in liver.

MATERIALS AND METHODS

Generation of PRIP Conditional Null Mutation in Liver (PRIP$^{Liv-/-}$)

Heterozygous LoxP-PRIP mice (41,66) were bred with albumin-Cre (AlbCre) transgenic mice (61) to delete the DNA fragment between LoxP1 and LoxP2 in the liver (66). The heterozygous mice with the expected deletion were interbred to generate homozygous mutants lacking PRIP in liver cells (PRIP$^{\text{Liv}-/-}$). Southern blot analysis of genomic DNA isolated from PRIP$^{+/+}$ and PRIP$^{\text{Liv}-/-}$ livers was performed to confirm deletion of targeted allele in PRIP$^{\text{Liv}-/-}$ livers. Genomic DNA was digested with *Spe*I and hybridized with a probe (probe 1) spanning part of intron 5 and exon 6 of the PRIP gene. To further confirm homozygosity of PRIP gene deletion, genomic DNA was hybridized with a probe (probe 2) designed within

exon 7 of the PRIP gene. Generation of PBP[Liv−/−] has been described elsewhere (16–18). Mice were housed in a pathogen-free animal facility under standard 12-h light/12-h dark cycle and maintained on standard rodent chow and water ad libitum. All animal procedures used in this study were reviewed and preapproved by the Institutional Review Boards for Animal Research of the Northwestern University.

Treatment With PPARα and CAR Agonists

For assessing the role of PRIP in PPARα ligand-induced changes in liver, groups of PRIP[Liv−/−] mice ($n = 5$–8) aged 5–6 weeks were fed powdered diet containing PPARα-ligand, Wy-14,643 (0.125% w/w), for 2 weeks (47). Groups of mice were treated with CAR ligand, phenobarbital (PB; 100 mg/kg body weight), or TCPOBOP (3 mg/kg body weight) by IP injection daily for 3 days (5,16). A single IP injection of acetaminophen (APAP) (250 mg/kg body weight) was given to mice pretreated with corn oil, PB, or TCPOBOP as described above, 24 h before sacrifice (16). To assess cell proliferation in liver, mice treated with TCPOBOP or Wy-14,643 were given bromodeoxyuridine (BrdUrd) (1.0 mg/ml) in drinking water for 4 days and analyzed immunohistochemically for liver BrdUrd nuclear-labeling indices.

Zoxazolamine Paralysis Test

Mice pretreated for 3 days with either corn oil or PB, as described above, were given a single IP injection of zoxazolamine (300 mg/kg body weight, Sigma), 24 h after the last dose of PB. Mice were placed on their backs and paralysis time was monitored (the time required for the animal to regain sufficient consciousness to right itself repeatedly) as described (16,53,56,65).

Adenovirally Driven Expression of EGFP-CAR

Mouse CAR cDNA was cloned into pEGFP-C1 vector (Clontech) and EGFP-CAR fragment was obtained by PCR as described (10). EGFP-CAR was cloned into pShuttle-CMV expression vector (Quantum Biotechnologies, Inc.) at XhoI and HindIII sites. The linearized shuttle vector and AdEasy vector (Quantum Biotechnologies, Inc.) were then cotransformed into Escherichia coli strain BJ5183. Positive recombinant plasmid Ad/EGFP-CAR was selected and the Ad/EGFP-CAR virus was then generated as described for Ad/PBP virus (16). For the expression of adenovirally driven EGFP-CAR, wild-type (C57BL/6J) and PRIP[Liv−/−] mice were injected intravenously (via tail vein) with 4×10^{11} recombinant ade-

novirus particles in a volume of 200 μl and sacrificed 5 days after injection. Phenobarbital (100 mg/kg body weight) or the solvent was injected IP 3 h prior to sacrifice. Small pieces of liver were used for cutting frozen sections, ~8 μm thick, fixed in 4% paraformaldehyde, and examined for fluorescence. Nuclear DNA was visualized by DAPI staining (10).

Mouse Primary Hepatocyte Culture and Adenovirus Infection

Hepatocytes were isolated from mouse livers using collagenase perfusion method as described previously (23). Cells were plated on collagen-coated 12-well plates containing William's E medium at a cell density of 2.5–3.0×10^5 cells/well. After 8-h or overnight culture, the cells were infected with Ad/EGFP-CAR at a multiplicity of infection of ~40. Twenty-four hours postinfection, cells were treated with or without ligand (1 mM phenobarbital). Cellular localization of CAR was assessed by fluorescence microscopy 2 h after the addition of CAR ligand, penobarbital.

Northern and Western Blot Analyses

Total RNA isolated using TRIzol reagent (Invitrogen) was glyoxylated, separated on an 0.8% agarose gel, transferred to nylon membrane, and probed with selected ^{32}P-labeled cDNAs (16,43). Equal loading of total RNA in the gel was verified by measuring the intensity of 18S and 28S rRNA bands stained with Fast RNA Stain (HealthGene Corp). Immunoblotting of liver homogenates was performed using antibodies against CAR (Santa Cruz Biotechnology and M. Negishi, National Institute of Environmental Health Sciences, Research Triangle Park, NC), GSTπ (Dako), GFP (Invitrogen), and catalase (16,43).

RT-PCR and Quantitative PCR

RT-PCR was performed with the SuperScript one-step RT-PCR kit from Invitrogen as instructed. Primers 5′-CCC AGG CCA GTT CAC AGC-3′ and 5′-GGC CTG TGG TGT CCA A-3′ were used to amplify the region flanking exon 7 in the PRIP homozygous mutant. Total RNA (1 μg) was reverse transcribed with SuperScript II reverse transcriptase followed by PCR amplification consisting of 35 cycles of denaturing at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min using a One-step RT-PCR kit (Invitrogen). For quantitative PCR, cDNA was synthesized from 2 μg total RNA using a Superscript III First Strand Synthesis System (Invitrogen) as per kit instructions. The PCR primers 5′-GCA AAT GCT

GCC ATA GCC TCT GG- 3′ and 5′-GCC GAA GCA GGT CGA GAG GAT C- 3′ were used to detect PRIP mRNA expression. qPCR was carried out in triplicates for the amplification of PRIP gene and expression values were normalized with 18S ribosomal rRNA levels. The PCR reaction consisted of 1 μl (100 μmol) of sense and antisense primers and 10 μl of 2× SYBR Green denaturation at 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C and 95°C for 15 s. The generation of specific PCR products was confirmed by melting curve analysis and relative gene expression changes were measured using the comparative C_T method, $X = 2^{-\Delta\Delta CT}$.

Immunohistochemical Analyses

Livers were fixed in either 10% formalin or 4% paraformaldehyde, and 4-μm-thick paraffin sections were cut and stained with hematoxylin and eosin. For immunohistochemical analyses, sections were deparaffinized and rehydrated. After inactivation of endogenous peroxidase activity and antigen retrieval, sections were blocked with 10% normal bovine serum in phosphate-buffered saline, followed by sequential incubation at room temperature with antibodies against CAR (M-150, SC-13065; Lot I2404, Santa Cruz Biotechnology) or PRIP for 3 h, biotinylated anti-rabbit IgG for 1 h, streptavidin-linked horseradish peroxidase (Vector laboratories) for 30 min, and finally 3,3′-diaminobenzidine tetrahydrochloride solution (Biogenex Inc.) for 4 min (16). BrdUrd incorporation was determined using a mouse anti-BrdUrd monoclonal antibody (DAKO) and Vectastain ABC Kit (Vector Laboratories).

Serum Alanine Aminotransferase (ALT) Assay

Blood collected from inferior vena cava was used for determination of serum (ALT) activity by using an ALT assay kit (Thermo Electron Corp.). Liver homogenates were used for GSH assay (Sigma) (16).

RESULTS

PRIP Liver Conditional (PRIP[Liv−/−]) Nulls

PRIP null mutation results in embryonic lethality that precludes investigations on the role of this coactivator in the adult liver (1,28,34,66). To circumvent this, mice with the LoxP integrated recombinant PRIP gene (LoxP-PRIP) were generated (41,66) and crossed with transgenic mice with the Cre transgene driven by the albumin promoter (Alb-Cre) (61). Alb-Cre transgenic mouse expresses Cre recombinase specifically in hepatic parenchymal cells but not in other cells in liver (16,17,61). Expression in liver of albumin promoter-driven Cre recombinase, commencing during the immediate postpartum stage with maximal expression occurring at around 2 weeks postnatally, results in the deletion of the exon 7 of the PRIP gene flanked by two LoxP sites (Fig. 1A). Deletion of exon 7 leads to a reading frame-shift resulting in the generation of a stop codon right after the fusion between exon 6 and exon 8. Deletion of PRIP gene in hepatocytes was confirmed by Southern blotting (Fig. 1B), RT-PCR (Fig. 1C), quantitative PCR (Fig. 1D), and immunohistochemical staining of liver sections for Cre and PRIP expression (Fig. 1E). As expected, Cre protein is absent in wild-type (PRIP^{+/+}) livers but is expressed in PRIP[Liv−/−] (Fig. 1E). On the other hand, PRIP nuclear staining is seen in PRIP^{+/+} wild-type livers but not in Cre expressing PRIP[Liv−/−], thus visually confirming that the deleted PRIP gene did not express the PRIP protein in liver (Fig. 1E).

PPARα and CAR Ligand-Induced Hepatocellular Proliferation

To evaluate the role of coactivator PRIP in PPARα and CAR ligand-induced liver cell proliferation, PRIP[Liv−/−] mice were treated with either Wy-14,643, a potent PPARα ligand, or with CAR ligands, phenobarbital or TCPOBOP (Fig. 2). Short-term treatment with either PPARα or CAR ligands increased liver weight/body weight ratios in both PRIP^{+/+} and PRIP[Liv−/−] mice (Fig. 2A). The hepatomegalic effect was more pronounced in both PRIP^{+/+} and PRIP[Liv−/−] mice treated with TCPOBOP and Wy-14,643, compared to phenobarbital treatment. Hepatocellular proliferation, as assessed by BrdUrd labeling indices, was prominent in the livers of both PRIP^{+/+} and PRIP[Liv−/−] following TCPOBOP and Wy-14,643 treatment. There was no difference in the extent of liver cell proliferation with either ligand in the presence or absence of PRIP expression (Fig. 2B, C). Figure 2C illustrates representative photomicrographs of liver sections immunohistochemically analyzed for BrdUrd incorporation. In untreated livers, BrdUrd labeling was seen in an occasional hepatocyte nucleus but the number of labeled hepatocytes was markedly increased throughout the liver lobule in PRIP^{+/+} and PRIP[Liv−/−] mice.

Induction of Peroxisome Proliferation and PPARα-Regulated Genes in Liver

The highly reproducible pleiotropic responses in the rat and mouse liver induced by peroxisome proliferators have been well documented (45). These structurally diverse chemicals act by functioning as ligands for the nuclear receptor PPARα and disruption
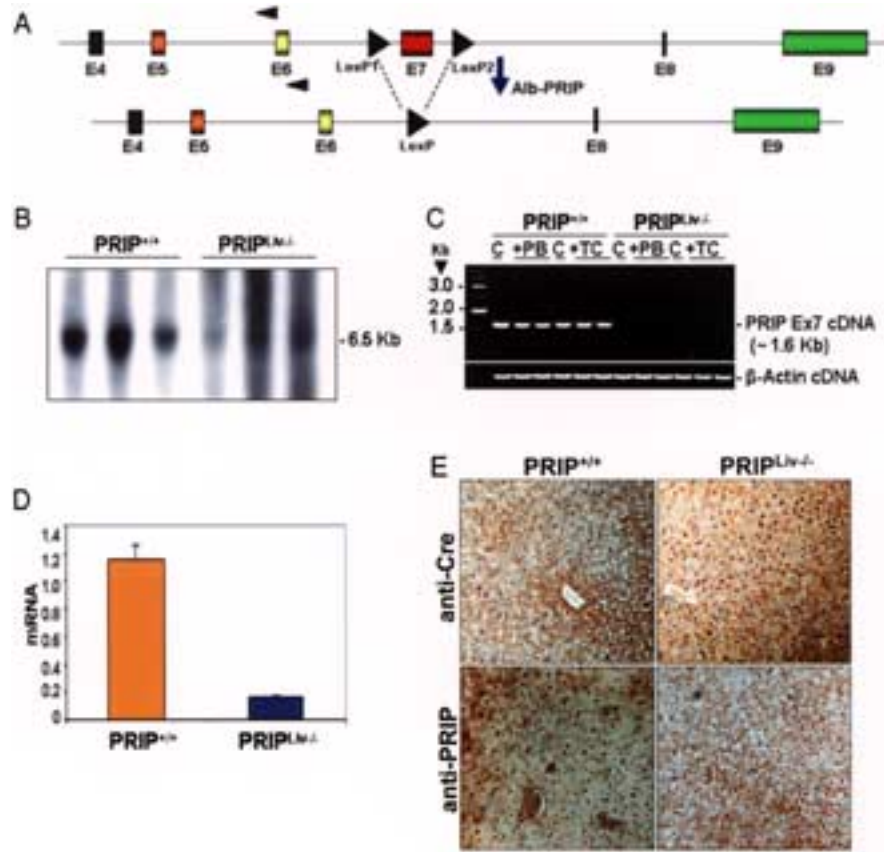
Figure 1. Conditional disruption of the PRIP gene in mouse liver. (A) Schematic diagram of LoxP-PRIP gene, with exon 7 flanked by LoxP sites and the resulting Cre-mediated deletion of exon 7. LoxP-PRIP mouse was crossed with an Alb-Cre mouse to produce LoxP-PRIP mice expressing Alb-Cre. Expression in liver of Cre recombinase is necessary for the deletion of the exon 7. (B) Genomic DNA from liver of wild-type (PRIP^{+/+}) and PRIP[Liv−/−] mice (3 each group) digested with *Spe*I and hybridized with exon 7 specific probe. A 6.5-kb band seen in PRIP^{+/+} mice is not apparent in PRIP[Liv−/−] mice. (C) Total RNA isolated from liver of PRIP^{+/+} or PRIP[Liv−/−] (C, control; PB, phenobarbital treated; TC, TCPOBOP treated) was used for RT-PCR amplification with primers specific for the LoxP-PRIP transgene and the deleted PRIP gene. An expected band was observed in wild-type PRIP^{+/+} mouse livers but not in PRIP[Liv−/−] livers. β-Actin served as a loading control. (D) Expression levels of PRIP mRNA in PRIP^{+/+} or PRIP[Liv−/−] livers as assessed by qPCR. mRNA levels were normalized to 18S rRNA levels. A mean of triplicates is shown (mean ± SE; $n = 3$). (E) Immunohistochemical staining of liver sections from PRIP^{+/+} and PRIP[Liv−/−] mice with anti-PRIP and anti-Cre antibodies. Cre staining of nuclei is seen in PRIP[Liv−/−] livers but not in wild-type PRIP^{+/+} livers, whereas PRIP nuclear staining is absent in Cre-expressing livers but present in the PRIP^{+/+} livers.

of PPARα gene abrogates these responses (14,29). To assess the role of PRIP in PPARα ligand-induced peroxisome proliferation and gene expression in liver, diet containing Wy-14,643 was fed for 2 weeks and livers were analyzed for morphological alterations and for the expression of selected PPARα target genes. Light microscopy of hematoxylin and eosin (H&E)-stained liver sections revealed hypertrophy of all hepatocytes in the liver lobule with granular eosinophilic cytoplasm in Wy-14,643-treated PRIP^{+/+} and PRIP[Liv−/−] mice compared to untreated controls (Fig. 3A). Evaluation of 0.5-μ-thick sections of liver that were processed for visualizing peroxisomal catalase showed a profound increase in the number of these catalase-positive organelles in all hepatocytes in both PRIP^{+/+} and PRIP[Liv−/−] mice (Fig. 3B). The degree of peroxisome proliferation in liver

cells, as assessed by light and electron microscopy (not illustrated), was essentially similar in both PRIP^{+/+} and PRIP[Liv−/−] mice. These observations suggest that the presence of coactivator PRIP is not essential for PPARα ligand-induced peroxisome proliferation in liver.

Analysis of RNA isolated from livers of untreated and peroxisome proliferator-treated mice for PPARα-regulated gene expression revealed no differences in the expression of key genes involved in peroxisomal β-oxidation and microsomal ω-oxidation (Fig. 4A). The hepatic mRNA levels of all three β-oxidation system genes, namely fatty acyl-CoA oxidase (AOX), enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (L-bifunctional enzyme; L-PBE), and peroxisomal thiolase (PTL), increased markedly in Wy-14,643-treated PRIP^{+/+} and PRIP[Liv−/−] mice. The
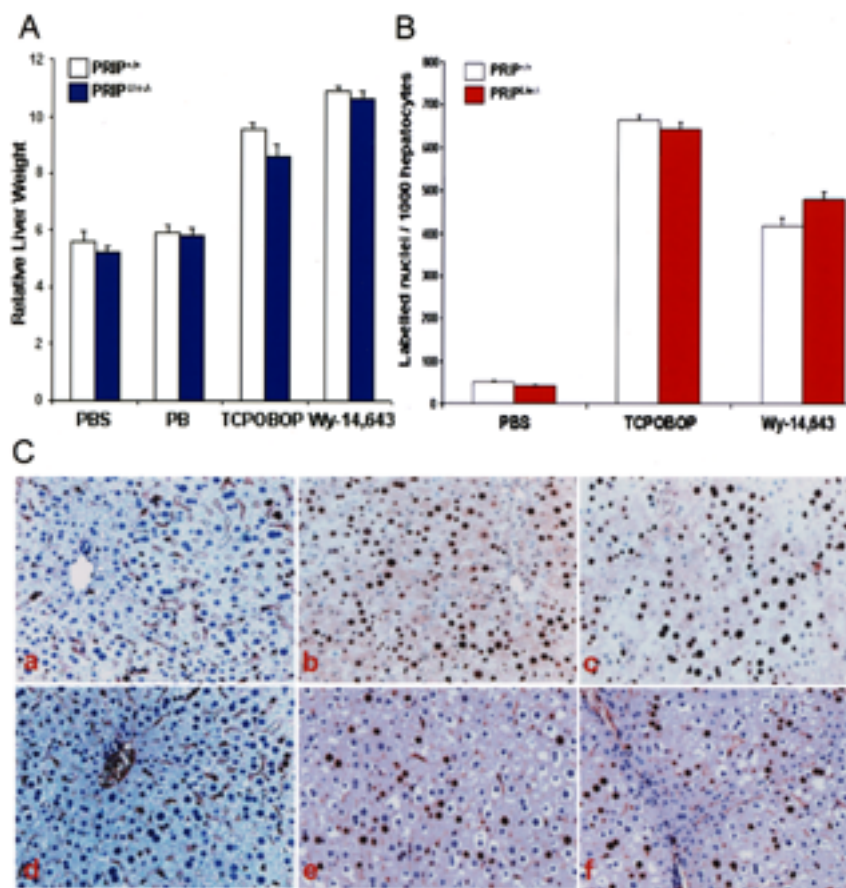
Figure 2. Changes in liver weight and hepatocellular proliferation. (A) Liver weight/body weight ratios in PRIP^{+/+} and PRIP[Liv–/–] mice treated IP with PB or TCPOBOP (TC) daily for 3 days, or fed Wy-14,643 (0.125%) in the diet for 4 days compared with PBS injected controls. (B) Hepatocellular BrdUrd labeling indices in TCPOBOP- and Wy-14,643-treated mice. BrdUrd was given in drinking water for 3 days (1.0 mg/ml) to mice given TCPOBOP for 3 days, or fed a diet containing Wy-14,643 for 4 days. Liver cell proliferation is similar for each compound in both PRIP^{+/+} and PRIP[Liv–/–]mice. (C) Representative photomicrographs to illustrate BrdUrd-labeled nuclei in the livers of untreated PRIP^{+/+} (a) and PRIP[Liv–/–] (d) controls, and TCPOBOP (b, c) and Wy-14,643 (e, f) treated PRIP^{+/+} (b, e) and PRIP[Liv–/–] (c, f) mice.

mRNA level of CYP4A1, which is responsible for microsomal cytochrome p450 fatty acid ω-hydroxylation, is also increased in these livers following treatment with a PPARα ligand (Fig. 4A). PEX-11, which is involved in peroxisome biogenesis, is also upregulated in livers with peroxisome proliferation. Catalase is a peroxisomal marker enzyme and treatment with peroxisome proliferators causes only a mild increase in its mRNA level. PPARα mRNA levels remain essentially similar in control and Wy-14,643-treated mice. Immunoblotting data confirm similar levels of increases in the amount of AOX, L-PBE, and PTL proteins in PRIP^{+/+} and PRIP[Liv–/–] mouse livers following Wy-14,643 treatment (Fig. 4B). The levels of D-bifunctional enzyme (D-PBE), and sterol carrier protein-x (SCP-x), the two enzymes involved in the branched chain fatty acid β-oxidation, increased only slightly in both types of mice. Immunoblotting also showed a visible but slight increase in PPARα and catalase proteins with Wy-14,643 treatment in PRIP^{+/+}

and PRIP[Liv–/–] mouse livers. These data further confirm that PRIP gene is not involved in the regulation of these genes.

PRIP Is Redundant for CAR-Regulated Gene Expression

In a previous brief report, we reported that induction of CYP genes in liver by CAR ligands, phenobarbital and TCPOBOP, is not dependent on coactivator PRIP. We now show that both these ligands induce CYP2B10, CYP3A11, CYP2C29, and CYP1A2 mRNA levels to a similar extent in both PRIP^{+/+} and PRIP[Liv–/–] mouse livers (Fig. 5A, B) and also include functional data showing that PRIP deletion does not affect zoxazolamine-induced paralysis (Table 1). PRIP deficiency does not affect the levels of expression of many genes analyzed by Northern blotting (Fig. 5A) and these include not only CYP genes but also three nuclear receptors, namely CAR, pregnane

X receptor (PXR), and farnesyl X receptor (FXR) (Fig. 5A). The mRNA expression of cytoplasmic CAR retention protein (CCRP) in liver appeared similar in treated and untreated groups (26). We also compared the mRNA expression levels of some key genes (CYP2B10, CYP3A11, CYP1A2) in the liver of PRIP[Liv−/−] and PBP[Liv−/−]mice treated with CAR ligands phenobarbital and TCPOBOP (Fig. 5B). The induction, for example, of CAR-regulated gene CYP2B10 was robust in PRIP-null livers but minimal in PBP-null livers (Fig. 5B). The slight increase in CYP2B10 mRNA in PBP livers following TCPOBOP treatment is due to residual presence of a small fraction of hepatocytes that escaped Cre-mediated gene deletion. Immunoblotting for CAR, GSTPi, and catalase showed similar levels of expression in all groups (Fig. 5C).

PRIP Deficiency Does Not Influence Zoxazolamine Paralysis and APAP-Induced Hepatic Necrosis

The functional significance of the induction of CAR-mediated transcription of CYP genes was examined by the assessment of paralysis induced by the muscle relaxant zoxazolamine and acetaminophen (APAP) hepatotoxicity (16,56). Induction of CYP1A2 by certain xenobiotics, including CAR ligand phenobarbital, leads to rapid metabolic inactivation of zoxazolamine, resulting in significant decrease in the duration of paralysis (16,56). We have previously reported that PBP[Liv−/−] mice exhibited significantly enhanced zoxazolamine sensitivity and phenobarbital pretreatment had no effect on zoxazolamine paralysis, confirming the functional significance of the failure of CYP gene induction in PBP-null livers (16). In the present study, PRIP gene deletion had no effect on the inducibility of CYP genes in liver and as a corollary the zoxazolamine paralysis was similar to that noted in wild-type (PRIP^{+/+}) mice (Table 1). We further examined the role of PRIP in APAP-induced hepatotoxicity. APAP hepatotoxic effects are influenced by a variety of factors that culminate in the conversion of APAP to its highly toxic intermediate metabolite, N-acetyl-p-benzoquinone imine (16,56, 65). It is well known that induction of CYP isoforms by activators of CAR or PXR enhances APAP hepatotoxicity whereas the disruption of CAR and PBP genes has an abrogating effect on this toxicity (6, 56,65). We now show that PRIP gene deletion had no abrogating effect on APAP hepatotoxicity (Fig. 6). Phenobarbital or TCPOBOP pretreatment resulted in the induction of expression of CYP genes in PRIP[Liv−/−] mouse liver and as a consequence developed centrilobular hepatic necrosis (Fig. 6A) due to rapid and efficient APAP metabolic conversion to the toxic metabolite, N-acetyl-p-benzoquinone imine (56). Serum ALT levels were high in both PRIP^{+/+} and PRIP[Liv−/−] mice pretreated with phenobarbital or TCPOBOP prior to APAP exposure (Fig. 6B). Administration of APAP alone without prior treatment with a CAR li-
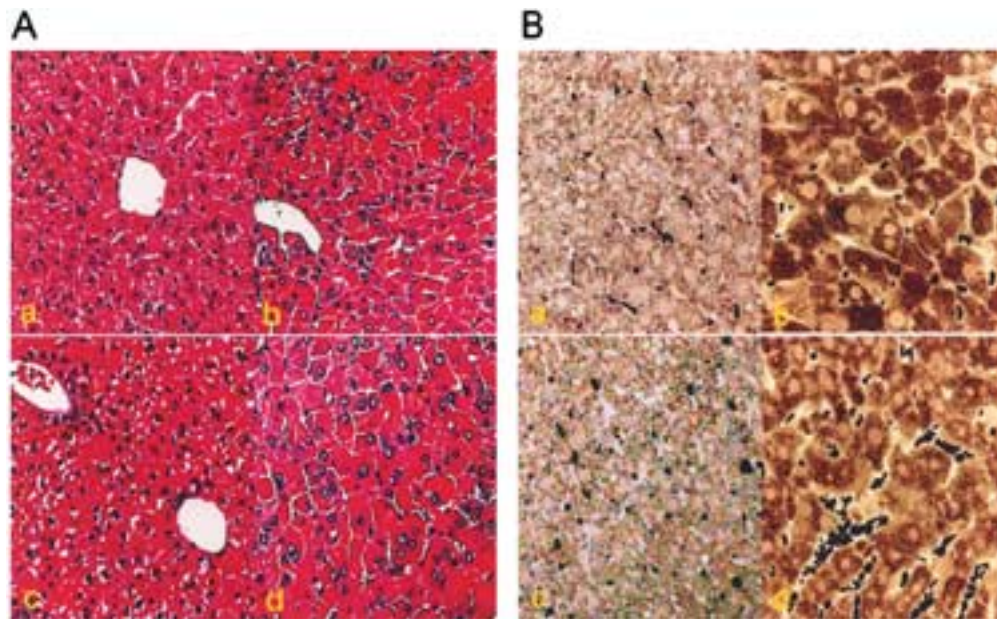

Figure 3. Comparative changes in liver morphology and cytochemical localization of peroxisomal catalase. (A) Liver morphology of PRIP^{+/+} (a, b) and PRIP[Liv−/−](c, d) mice treated with Wy-14,643 (b, d) for 7 days (H&E stained). (B) Peroxisome proliferation in hepatocytes of wild-type and PRIP[Liv−/−] mice treated with Wy-14,643 for 7 days. A massive increases increase in peroxisomes is evident in the livers of wild-type and PRIP[Liv−/−] mice treated with Wy-14,643 (b, d).
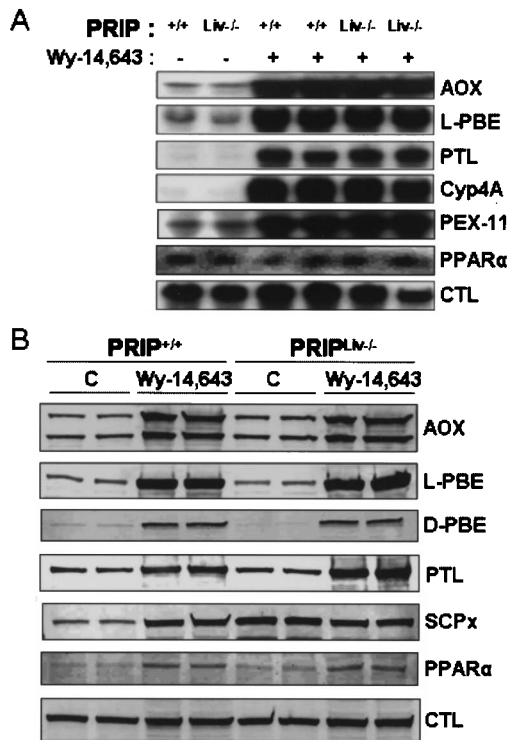
Figure 4. Expression of selected PPARα regulated genes in Wy-14,643-treated PRIP+/+ and PRIPLiv−/− mouse liver. (A) Northern blot analysis of total liver RNA from Wy-14,643-treated and untreated PRIPLiv−/− and wild-type (PRIP+/+) mice for peroxisomal β-oxidation system genes (AOX, L-PBE, PTL), microsomal ω-oxidation (CYP4A3) gene, and others. Peroxisomal marker enzyme catalase (CTL) is also included. (B) Immunoblot analysis of liver proteins of untreated and Wy-14,643-treated wild-type (+/+) and PRIPLiv−/−(Liv−/−) mice for AOX (two bands represent AOX components A (72 kDa) and B (51 kDa)), L-PBE, D-PBE, PTL, SCPx, PPARα, and CTL.

gand did not result in hepatic necrosis. We also measured GSH content in liver to ascertain if hepatotoxicity correlates with diminished GSH content. Decrease in GSH level occurred in both PRIP+/+ and PRIPLiv−/− mouse livers with APAP-induced hepatic necrosis (Fig. 6C).

PRIP Is Not Needed for Phenobarbital-Mediated Nuclear Translocation of CAR

Nuclear receptor CAR is classified as a type I nuclear receptor, because it is cytoplasmic in location and undergoes nuclear translocation after ligand binding (13,20–22,27,31,64). Upon entering the nucleus, the agonist bound receptor interacts with the LXXLL (L, leucine; X, any amino acid) nuclear receptor (NR) box of coactivators to assemble the transcriptional complex for initiation of transcription (11,48). Immunohistochemical staining for CAR in liver sections of wild-type (PRIP+/+) and PRIPLiv−/− mice treated with phenobarbital revealed translocation of endogenous

CAR into the nucleus of hepatocytes in contrast to the absence of CAR nuclear staining in untreated controls (Fig. 7A). Recently, we generated adenovirally driven EGFP-CAR vector for expression of CAR in liver in vivo and in hepatocytes in vitro. Expression of exogenous Ad/EGFP-CAR in liver was prominent in liver cells and, in the absence of CAR ligand, the EGFP-CAR was in the cytoplasm (Fig. 7B,D). Rapid translocation of CAR into the nucleus occurred within a short time after phenobarbital administration in both PRIP+/+ and PRIPLiv−/− livers (Fig. 7B–E). We also studied nuclear translocation of Ad/EGFP-CAR in primary hepatocyte cultures (Fig. 8). EGFP-CAR appeared cytoplasmic in wild-type (PRIP+/+) and PRIPLiv−/− mouse hepatocytes in culture in the absence of CAR ligand, and with phenobarbital CAR translocated to the nucleus irrespective of the PRIP status.

DISCUSSION

In this study, we report the generation of PRIP liver conditional null mice (PRIPLiv−/−) to study the role of this coactivator in the transcriptional regulation of genes controlled by nuclear receptors PPARα and CAR. We demonstrate that coactivator PRIP is not essential for the function of these two important xenobiotic sensing nuclear receptors. First, absence of PRIP in liver had no effect on PPARα ligand-induced hepatomegaly, hepatic peroxisome proliferation, and the induction of genes regulated by PPARα that participate in peroxisomal and microsomal fatty acid oxidation in liver. Second, the absence of PRIP in liver failed to abrogate CAR ligand-induced induction of certain CYP family genes involved in xenobiotic metabolism (53,56,57). Consequently, PRIP-null livers pretreated with CAR ligand phenobarbital showed hepatocellular necrosis when exposed to acetaminophen. In an earlier study, we demonstrated that steroid receptor coactivator-1 (SRC-1), a member of the p160/SRC-1 family of coactivators, is not essential for PPARα-regulated gene expression and induction of peroxisome proliferator-induced pleiotropic responses in mouse liver (43). SRC-1 gene deletion also exerted no effect on phenobarbital-mediated nuclear translocation of CAR and the induction of CAR-regulated genes in liver (D. Guo unpublished). Thus, it appears that coactivators SRC-1 and PRIP are redundant for the transcriptional activity of both PPARα and CAR, despite the fact that SRC-1 has intrinsic histone acetyltransferase activity needed for chromatin modification (8,48). In contrast, studies with PBP liver conditional null mice (PBPLiv−/−) established that coactivator PBP/MED1 deficiency in vivo
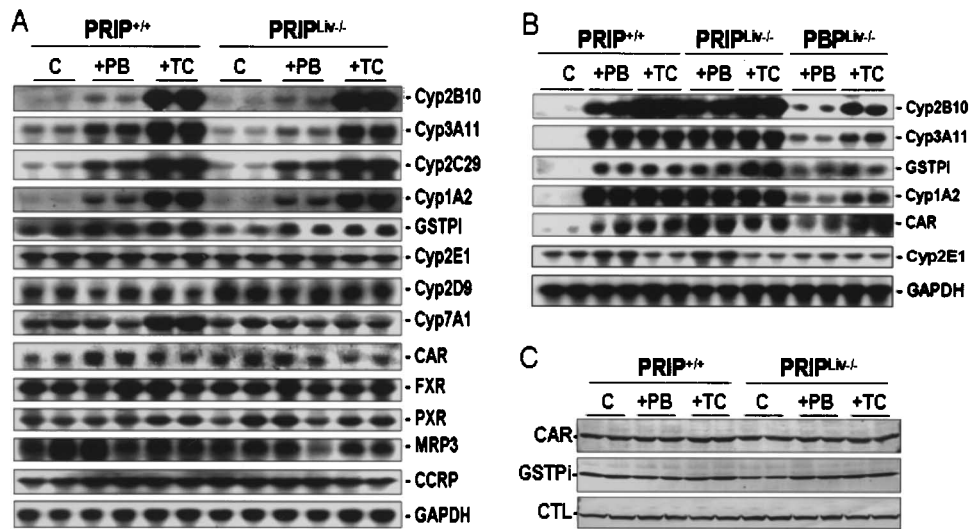
Figure 5. Changes in PB- and TCPOBOP-induced CAR target gene expression in liver. (A) Northern blot analysis of liver RNA from wild-type (PRIP$^{+/+}$) and PRIP$^{Liv-/-}$ mice given saline (con), phenobarbital (PB), or TCPOBOP (TC) injections for 3 days. The cDNA probes used for the analysis are indicated. Increases in Cyp2B10, Cyp3A11, Cyp2C29, and Cyp1A2 mRNA levels in wild-type and PRIP-null livers by PB or TCPOBOP appear similar. No differences in the expression levels of CAR, FXR, PXR, and CCRP are seen among different groups. GAPDH is used for assessing RNA loading. (B) Northern blot analysis for the changes in the expression of selected genes in PRIP$^{Liv-/-}$ and PBP$^{Liv-/-}$ mouse livers. Robust induction of CYP2B10 is seen in PRIP$^{Liv-/-}$ but not in PBP$^{Liv-/-}$ livers. (C) Immunoblot analysis of liver proteins for CAR, GSTPi, and catalase (CTL) in PB- and TCPOBOP (TC)-treated PRIP$^{+/+}$ and PRIP$^{Liv-/-}$ mice.

mimics the absence of PPARα, implying that PBP is required for PPARα-regulated gene expression in liver (17). Furthermore, PBP gene deletion in liver abrogated acetaminophen hepatotoxicity similar to that observed in CAR-null mouse liver (65). Accordingly, while PBP is an essential coactivator for PPARα and CAR function, both PRIP and SRC-1 are

TABLE 1
ZOXAZOLAMINE PARALYSIS TEST

	PRIP$^{+/+}$	PRIP$^{Liv-/-}$
Control	4/13: 2-h paralysis; recovered	1/11: 2-h paralysis; recovered
	5/13: 3-h paralysis; recovered	6/11: 3-h paralysis; recovered
	1/13: 4-h paralysis; recovered	1/11: 4-h paralysis; dead
	1/13: 6-h paralysis; recovered	1/11: 8-h paralysis; dead
	1/13: 6-h paralysis; dead	2/11: 12-h paralysis; recovered
	1/13: 9-h paralysis; dead	
PB (3 days)	8/8: not paralyzed	8/8: not paralyzed

Control and 3-day PB-treated male mice (5–6 weeks old) were given a single IP injection of zoxazolamine (300 mg/kg of body weight), and paralysis duration was recorded as the time when the mice were able to right themselves repeatedly. Very similar results were observed in both wild-type (PRIP$^{+/+}$) and knockout (PRIP$^{-/-}$) mouse groups.

considered redundant for the transcriptional activation of these two nuclear receptors.

The mechanism by which PBP/TRAP220 affects PPARα- and CAR-mediated transcriptional activation of their respective target genes is not fully known, but it appears that absence of PBP in liver cells diminishes the recruitment or association of other cofactors (16,17). The finding that both PPARα and CAR target gene expression mediated by cognate ligands in PRIP-null livers is similar to that noted in the wild-type mouse livers implies that coactivator assembly necessary for the function of these two nuclear receptors proceeds properly in the absence of PRIP. Furthermore, CAR is a cytoplasmic protein and it has to be translocated to the nucleus for transcriptional activity (26,36,38,40,50,53,58). We noted that the absence of PBP hinders the phenobarbital-mediated nuclear translocation of CAR or that PBP is required for retention of CAR in the nucleus (10,16). In an unliganded state, CAR is retained in the cytoplasm by complexing with Hsp90, and cytoplasmic CAR-retention protein (CCRP) (26,27,38). Phenobarbital-like CAR activators facilitate the recruitment of an okadaic acid-sensitive phosphatase to the CAR-Hsp90-CCRP complex, implying that CAR translocation may be phosphorylation dependent (53). Our results with PBP-null livers suggest that within the nucleus, imported CAR most likely binds to PBP and that PBP functions as a scaffolding pro-
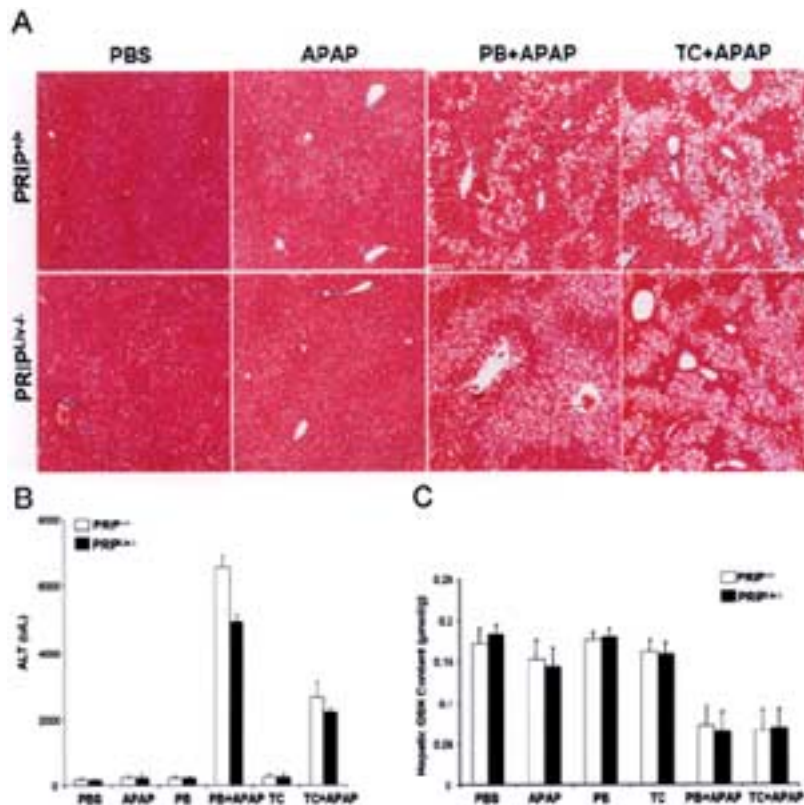
Figure 6. PRIP and CAR in APAP toxicity. (A) Histological appearance of livers from wild-type (PRIP^{+/+}) and PRIP[Liv-/-] mice treated with PBS, APAP, PB plus APAP, or TCPOBOP (TC) plus APAP (H&E staining). Centrilobular hepatic necrosis is prominent in wild-type as well as PRIP[Liv-/-] mice pretreated with PB or TCPOBOP (TC) prior to APAP administration. Administration of PBS or APAP alone showed no hepatic necrosis. (B) Serum ALT levels in mice. ALT levels increased significantly in PB- or TCPOBOP-pretreated PRIP^{+/+} and PRIP[Liv-/-] mice given APAP. (C) Hepatic GSH levels. Both PRIP^{+/+} and PRIP[Liv-/-] mice treated for 3 days with PB or TCPOBOP (TC) prior to APAP showed significantly reduced levels of hepatic GSH compared to GSH levels in PBS, APAP, PB, or TCPOBOP treatment.

tein to retain CAR. Robust binding of CAR to PBP containing two LXXLL motifs has been demonstrated, indicating a strong affinity between these two molecules (10). In the absence of this PBP scaffold, the influxed CAR is unlikely to be retained and concentrated within the nucleus (10,58). The fact that CAR is translocated to the nucleus and upregulates the expression of its target gene in the absence of coactivator PRIP is a clear indication that PRIP is not essential for the function of CAR. These observations clearly establish that coactivator PBP is required for CAR and PPARα function and induction of genes responsible for xenobiotic metabolism, whereas SRC-1 and PRIP appear not essential for the functioning of PPARα and CAR (16). SRC-1 and PRIP may be indirectly associated with PBP scaffold but the signal for PPARα- and CAR-driven transcription does not involve these two coactivators. Ligand-independent translocation of CAR into the nucleus was shown to occur in vivo in response to SRC-2/GRIP1 and expression of GRIP1 increased CAR-mediated transactivation about twofold (36,58). Recent work with

SCR-1-null livers showed that it is not required for CAR nuclear translocation and it would be important to examine the role of SRC-2 and SRC-3 and combinations of these molecules using single and double knockout mice (43,55,59,60).

PPARα and CAR ligands induce hepatocellular proliferation during the acute exposure phase (5,45, 47). Wy-14,643, a potent PPARα ligand, exerts a profound primary liver cell proliferating effect in mice (49) and this effect is abolished in mice deficient in PPARα (29) or coactivator PBP/MED1 (17). Likewise, CAR ligand TCPOBOP is a potent hepatomitogen and its mitogenic potential is severely curtailed in mice with conditional deletion of coactivator PBP (5,16). In the present study, conditional deletion of PRIP gene in liver had no adverse effect on hepatocellular proliferation induced by PPAR and CAR ligands. Although lack of PPARα completely abrogates the hepatocyte proliferation in response to peroxisome proliferators, the absence of PPARα enhanced hepatocyte proliferation in response to CAR ligand TCPOBOP (5). These observations suggest a
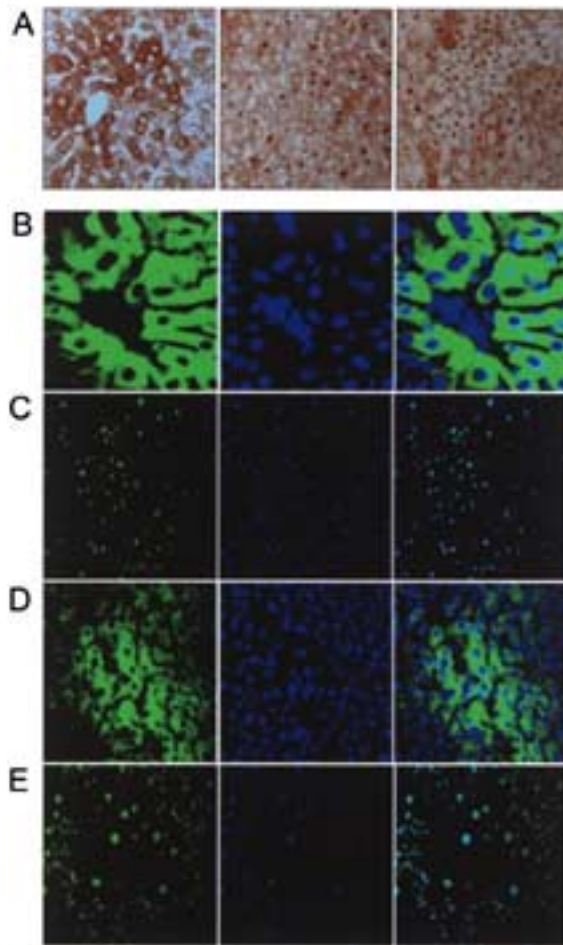
Figure 7. Absence of coactivator PRIP does not influence pheno-barbital-mediated nuclear translocation of CAR in mouse liver. (A) Immunoperoxidase staining of liver sections of wild-type control mouse (left panel), wild-type mouse treated with PB (middle panel), and PRIP[Liv−/−] mouse (right panel) treated with PB for localization of CAR. CAR was cytoplasmic in the untreated wild-type mouse whereas PB treatment caused nuclear translocation of CAR in wild-type as well as PRIP[Liv−/−] mouse livers. (B–E) Localization of exogenous Ad/EGFP-CAR in liver. EGFP fluorescence (left panels), DAPI staining (middle panels), and merged images (right panels) of wild-type (B, C) and PRIP[Liv−/−] (D, E) mice without (B, D) and with PB (C, E) treatment. PB induced nuclear translocation of Ad/EGFP-CAR in wild-type (C) and PRIP[Liv−/−] (E) mouse livers.

possible role for PPARα in CAR signaling (5). It is possible that absence of PPARα increases the RXR pool for CAR/RXR heterodimerization, resulting in enhanced cell proliferation (5). Both Wy-14,643 and TCPOBOP increased hepatocellular proliferation in PRIP^{+/+} and PRIP[Liv−/−] mice unlike that noted in the livers of mice deficient in PBP (16). These results show that PRIP does not play a role in the mitogenic effects mediated by PPARα and CAR ligands.

PRIP has been shown to enhance the transcriptional activation by CAR in the transfection assays in vitro, implying that PRIP serves as a coactivator for this xenobiotic receptor (4). PRIP also binds to CAR and this binding encompasses the first LXXLL motif of PRIP (4). These in vitro observations clearly imply that PRIP is involved in CAR-mediated gene transcription. However, the in vivo data obtained with PRIP[Liv−/−] mice in the present study failed to support the notion that PRIP is needed for the function of CAR. While PRIP amplifies CAR-mediated transcription in vitro, it appears that other factors may dominate in vivo, and supersede PRIP, making it redundant for CAR function. PRIP deficiency in liver failed to alter acetaminophen-induced hepatotoxicity in mice pretreated with CAR ligands PB or TCPOBOP. No differences in the upregulation of cytochrome P450 gene expression (CYP1A2, CYP2B10, CYP3A11, and CYP2E1) were seen following PB or TCPOBOP treatment between PRIP^{+/+} and PRIP[Liv−/−] mice. Moreover, CAR mRNA and protein levels were unchanged in PRIP[Liv−/−] mouse liver, suggesting that PRIP does not alter hepatic CAR expression. The mRNA levels of CCRP were also unchanged in PRIP-null livers. Functional analysis revealed that paralysis induced by zoxazolamine and hepatic necrosis induced by APAP were similar in PRIP[Liv−/−] and PRIP^{+/+} mice. These data firmly establish that PRIP deficiency in the liver does not protect the animal from the hepatotoxic effects of acetaminophen, unlike that seen with PBP deficiency (16). However, these findings are somewhat contradictory to the observations in transgenic mice expressing DN1, a fragment of PRIP/ASC-2 containing the first LXXLL motif (4). These mice failed to show acetaminophen-induced hepatic necrosis, suggesting that PRIP/ASC-2 is a bona fide coactivator for CAR. These differences are attributes to DN1 containing a functional LXXLL motif and this motif conceivably suppressed the function of other critical coactivators for CAR function such as PBP (16). Our studies on liver-specific PRIP-null mice demonstrate a more specific and reliable approach wherein the function of only one gene (PRIP) is disrupted as opposed to the dominant negative approach, which might have a more general effect and exert a broader dysfunction. Our findings with PRIP deficiency are unlike those obtained with PBP deficiency with regard to transcriptional activation of PPARα- and CAR-regulated genes (16,17). The reason for this selective dependence of PPARα- and CAR-regulated gene transcription on coactivator PBP but not on PRIP may be due to the existence of coactivator selectivity in nuclear receptor function (2,3,8, 11,35).

We thus conclude that transcription coactivator PRIP is not essential for induction of the hepatic effects of xenobiotic receptors CAR and PPARα. Nonetheless, whole body knockout of this gene is
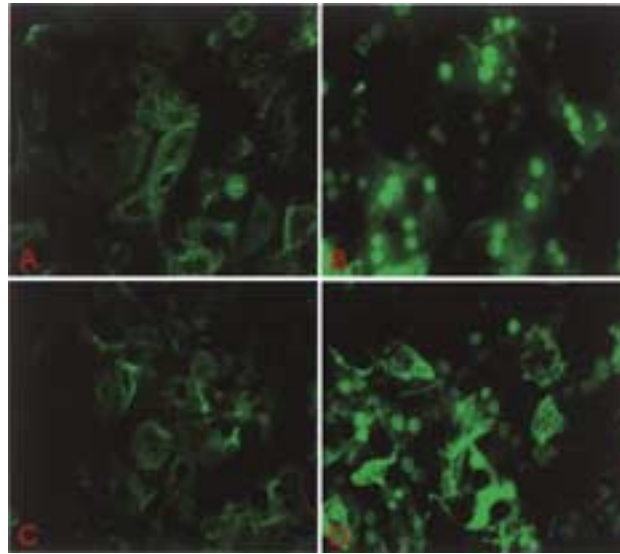
Figure 8. Nuclear translocation of Ad/EGFP-CAR in primary hepatocyte cultures. (A, B) Wild-type mouse hepatocytes in culture without (A) and with phenobarbital (B) in the medium. (C, D) PRIP$^{Liv-/-}$ mouse hepatocytes without (C) and with (D) PB in culture medium. CAR is mostly cytoplasmic in the absence of PB (A, C) and nuclear (B, D) following PB.

embryonic lethal, implying that this is an essential coactivator and that it may be involved in the function of nuclear receptors and transcription factors other than CAR and PPARα. Additional studies are necessary to explore in detail the selectivity and specificity of coactivator requirement for transcription of a given nuclear receptor.

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