Phenobarbital (PB) induction of CYP2B genes is mediated by translocation of the constitutively active androstan e receptor (CAR) to the nucleus. Interaction of CAR with p160 coactivators and enhancement of CAR transactivation by the coactivators have been shown in cultured cells. In the present studies, the interaction of CAR with the p160 coactivator glucocorticoid receptor-interacting protein 1 (GRIP1) was examined in vitro and in vivo. Binding of GRIP1 to CAR was shown by glutathione S-transferase (GST) pull-down and affinity DNA binding. N- or C-terminal fragments of GRIP1 that contained the central receptor-interacting domain bound to GST-CAR, but the presence of ligand increased the binding to GST-CAR of only the fragments containing the C-terminal region. In gel shift analysis, binding to CAR was observed only with GRIP1 fragments containing the C-terminal region, and the binding was increased by a CAR agonist and decreased by a CAR antagonist. Expression of GRIP1 enhanced CAR-mediated transactivation in cultured hepatic-derived cells 2-3-fold. In hepatocytes transsected in vivo, expression of exogenous GRIP1 alone induced transactivation of the CYP2B1 PB-dependent enhancer 15-fold, whereas CAR expression alone resulted in only a 3-fold enhancement in untreated mice. Remarkably, CAR and GRIP1 together synergistically transactivated the enhancer about 150-fold, which is approximately equal to activation by PB treatment. In PB-treated mice, expression of exogenous CAR alone had little effect, expression of GRIP1 increased transactivation about 2-fold, and with CAR and GRIP1, a 4-fold activation was observed. In untreated mice, expression of GRIP resulted in nuclear translocation of green fluorescent protein-CAR. These results strongly suggest that a p160 coactivator functions in CAR-mediated transactivation in vivo in response to PB treatment and that the synergistic activation of CAR by GRIP in untreated animals results from both nuclear translocation and activation of CAR.

In response to treatment with drugs or other xenobiotics, metabolism of the administered drug or other drugs is often increased (1). Underlying the increase in most cases is an induction of the expression of cytochrome P450 genes. Different subsets of cytochrome P450 genes are induced by different chemicals. Recently, members of the nuclear receptor family that form heterodimers with RXR, including peroxisomal proliferator activating receptor α, pregnane X receptor/steroid X receptor, and CAR, have been identified as mediators of the cellular response to xenobiotics (reviewed in Ref. 2). These nuclear receptors have relatively low specificity and affinity for their ligands so that they can be activated by a wide range of structurally diverse chemicals and thus comprise a broad response mechanism to xenobiotics.

CAR has been identified as the mediator of induction of CYP2B genes by the classical inducer of drug metabolism, PB. CAR was implicated in PB induction of CYP2B genes by the observation that CAR was selectively present in nuclear extracts from PB-treated animals and could bind to site with direct repeats separated by 4 base pairs (NR-1 and NR-2) in the CYP2B PB-responsive enhancer, termed PBRE or PB-responsive module (3). In cultured cells, expression of CAR by transient or stable transfection could transactivate the PBRE or induce the expression of the endogenous CYP2B6 gene in HepG2 cells (4–7). The loss of PB induction of Cyp2b genes in transgenic mice with a disrupted CAR gene provided conclusive evidence of an essential role for CAR in PB induction (8).

CAR is unusual among the nuclear receptors in that it has relatively high constitutive activity (9). The initial ligands identified for CAR, androstanes, inhibited rather than activated CAR so that CAR was considered constitutively active (10). The concentrations of androstanes required for inhibition were higher than physiological concentrations, so it was initially unclear how PB induction could be mediated by such a constitutive nuclear receptor. This was clarified by the observation that CAR in untreated animals or primary cultures of hepatocytes is predominantly located in the cytoplasm of hepatocytes in contrast to continuously cultured cells where it is located in the nucleus (11). Treatment with PB resulted in translocation into the nucleus, which should be sufficient for transactivation of the PBRE because of its constitutive activity. Binding of PB to CAR was not detected, but binding of other PB-like ligands, such as TCPOBOP, was detected by several
techniques, and activation of CAR by these ligands was implied by an increase in interaction with the coactivator SRC-1 in the presence of TCPOBOP (12, 13). This led to a two-stage model for CAR activation in which (i) translocation into the nucleus was induced by PB-like inducers and (ii) CAR was directly activated by some of the inducers. The translocation was inhibited by okadaic acid, suggesting that phosphatase activity is required for the translocation (11), and activation in the nucleus could be blocked by a Ca²⁺/CaM-dependent protein kinase inhibitor, suggesting a role for phosphorylation in the activation (14).

The mechanism by which CAR transactivates the PBRU is not clear. In transient transfections, in addition to the NR-1 and NR-2 CAR-binding sites, a nuclear factor 1 site between and sequences flanking these NR sites are required for maximal PB induction (4, 15–17). Like other nuclear receptors, CAR transactivation probably involves coactivator proteins. The p160 coactivator SRC-1 has been shown to bind to CAR both biochemically and in two-hybrid studies, and the binding was decreased by antagonists, androstanes, and increased by the agonist, TCPOBOP (10, 12). In primary cultures of hepatocytes, SRC-1 expression alone increased transactivation of the PBRU but not a synthetic enhancer with two of the CAR NR-1 sites about 3-fold in untreated cells and similarly increased transactivation 2–3-fold in cells expressing exogenous CAR (7). These results suggest that p160 coactivators interact with and enhance transactivation by CAR, but the relatively small increases in transactivation mediated by overexpressed SRC-1 and the assay of activity in cultured hepatocytes in which the relative concentrations of regulatory factors may differ from hepatocytes in vivo fall short of establishing a role for these coactivators in PB induction.

SRC-1 is a member of a related p160 coactivators that includes SRC-1, TIF2/GRIP1, and RA3/ACTR/pCIP/P/ AIB-1 (18). GRIP1 has been shown to potentiate hepatic nuclear receptors, for example hepatic nuclear factor 4 (19, 20), and thus is a potential coactivator for CAR in the liver. We now show that GRIP1 interacts with CAR and with DNA-bound CAR-RXR and that the binding is modulated by ligands. GRIP1 modestly enhances CAR-mediated activation in continuously cultured cells. Remarkably, in untreated mice, exogenous expression of GRIP1 in hepatocytes transfected in vivo increases transactivation of the PBRU more than expression of CAR does, and coexpression with CAR results in a dramatic synergistic activation equal to that resulting from PB treatment. In PB-treated animals, exogenous expression of GRIP1 also results in a 2-fold increase in transactivation, whereas CAR has little effect and a 4-fold increase is observed if both exogenous CAR and GRIP are expressed.

MATERIALS AND METHODS

Plasmids—Vectors for expression of CAR in mammalian cells (pcDNA3-CAR) and bacteria (pETCAR) have been described (6). For expression of GST-CAR, a BamH1/EcoR1 fragment containing the CAR cDNA was inserted into pGEX2TK digested with the same enzymes. The expression vector, pG55.HA-GRIP1, encoding full-length GRIP1 and the bacterial expression vectors pGEX.GRIP1, pGEX.GRIP2, pGEX.GRIP3A, pGEX.GRIP3B, pGEX.GRIP5, and pGEX.GRIP6, were obtained from M. R. Steml. The vector pCMX-RXR was obtained from R. Evans. The expression vector for GFP-CAR, pEGFP-CAR1, was constructed by inserting a BamHI/EcoR1 fragment from pcDNA3-CAR containing the CAR cDNA into the BglII/EcoR1 site of pEGFP-C1 (BD Biosciences Clontech). Expression and Purification of CAR, RXR, and GRIP1—His-tagged CAR and FLAG-tagged RXR were expressed in bacteria and isolated as described (6).

GST Pull-downs—GST fusion proteins were expressed in Escherichia coli BL21(DE3) (pLys) and purified by binding to glutathione-Sepharose (Pharmacia Corp.) according to the manufacturer’s protocols. 35S-labeled proteins were synthesized by transcription of the mRNA and translation in reticulocyte lysates (Promega Corp.) according to the manufacturer’s instructions, and the lysate was precleared by incubation with GST bound to glutathione-Sepharose for 30 min at 4 °C. One μg of GST or GST fusion proteins bound to glutathione-Sepharose were incubated at 4 °C for 2 h with 4 μl of precleared lysate containing the 35S-labeled proteins in 200 μl of binding buffer (25 mM KOH-HEPES, pH 7.6, 100 mM NaCl, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mg/ml BSA, and 0.05% Nonidet P-40). In some cases, 5 μl TCPOBOP or 50 μl androstenediol dissolved in Me2SO or Me2SO alone (0.25% volume) were added to the reactions. After incubation, the samples were washed by centrifugation and resuspension in binding buffer without BSA five times. The supernatants were eluted from the Sepharose by incubation with 30 μl reduced glutathione in the same buffer, the eluted proteins were separated by SDS-PAGE, and the radioactive proteins were detected by autoradiography.

Affinity DNA Binding—A biotinylated DNA fragment containing four copies of the CYP2B1 NR1 site was synthesized by PCR using biotinylated oligonucleotide primers. CAR and RXR were purified from the agarose-bound labeled probes as described (6). CAR and GRIP were expressed in bacteria and isolated as described (6). For transfections, the cells were seeded in 24-well plates. HepG2 cells were cultured and transfected using LipofectAMINE as described (6). The DNA probe was a 32P end-labeled oligonucleotide containing the CYP2B1 NR-1 site, and 5,000–10,000 cpm were added to each reaction. 5 μl TCPOBOP, 50 μl androstenediol, or 1 μM 9-cis-retinoic acid was added to the binding reactions as indicated under “Results.”

Cell Culture and Transfection—Mouse Hepa1c1c7 and human HepG2 cells were cultured and transfected using LipofectAMINE as described (6). For transfections, the cells were seeded in 24-well plates. 1 μg/well of reporter plasmid containing either the CYP2B1 PBRU or four copies of the NR-1 site from the PBRU fused to the minimal CYP2C1 promoter and firefly luciferase reporter gene, 10 ng/well of pRL-SV40 plasmid DNA, containing the SV40 promoter and Renilla luciferase gene, and varying amounts as indicated of expression vectors for CAR and GRIP1 were added to each well. In experiments in which androstenediol or TCPOBOP were added, the cells were incubated for 24 h after transfection. Fresh medium containing the ligands was added, and the cells were incubated for an additional 24 h. The luciferase activities were determined by the dual luciferase reporter assay system (Promega Biotech), and the firefly luciferase values were normalized to the Renilla values for each sample.

Tail Vein Injection—Plasmid DNAs used for injection into mouse tail veins were purified by two rounds of CsCl density gradient centrifugation. 6–8-week-old (20–25 g) BALB/c male mice (Harlan Labs) were injected via the tail vein using the TransIT In Vivo Gene Delivery Reagent as described by the manufacturer (Promega Biotech), and the firefly luciferase values were normalized to the Renilla values for each sample.

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**GRIP1 Mediates Translocation and Activation of CAR in Vivo**

**RESULTS**

**GRIP1 Interacts with CAR**—To determine whether GRIP1 interacts with CAR in vitro, the binding of 35S-labeled GRIP1 to GST-CAR was analyzed by GST pull-downs. Full-length GRIP1 was bound to GST-CAR, whereas little or no binding of GRIP1 to GST was observed (Fig. 1B). The CAR antagonist androstenol (10) had little effect on the binding, whereas the agonist TCPOBOP (12) increased the binding. In the converse experiment with 35S-labeled CAR and GST-GRIP1, three fragments of GRIP1, GRIP1-1 (5–766), GRIP1-2 (530–1121), and GRIP1-3 (730–1121) fused to GST were examined (Fig. 1A). GRIP1 contains three LXXLL sequences within a central nuclear receptor-interacting domain, which extends from amino acids 641 to 749 (21). GRIP1-1 and GRIP1-2 contain all three LXXLL motifs in this domain, whereas GRIP1-3 contains only the third motif. Strong binding of 35S-CAR was observed to GRIP1-1 (Fig. 1C, lane 2), and weaker binding was observed for the other two fragments (Fig. 1C, lanes 5 and 8), whereas no binding to GST was observed (Fig. 1C, lane 11). Interestingly, the CAR ligands had little effect on binding to GRIP1-1, but androstenol modestly decreased, and TCPOBOP increased the binding of CAR to GRIP1-2 and GRIP1-3. These results are consistent with binding of GRIP1 to CAR through the central nuclear receptor-interacting domain and suggest that changes in interaction of GRIP1 with CAR by ligand binding are mediated by the C-terminal region.

**GRIP1 Interacts with DNA-bound CAR-RXR**—Although the previous results showed that GRIP1 could interact with CAR, to mediate transactivation of CAR, GRIP1 must bind to CAR when it is part of a heterodimer with RXR and is bound to DNA. Binding of CAR to RXR and to DNA could either mask binding sites for GRIP1 that are observed when binding to CAR alone is studied or alter the conformation of CAR so that binding of GRIP1 is altered. Further, GRIP1 has been shown to interact with RXR (22), so that GRIP1 potentially could interact with both CAR and RXR in an additive or synergistic manner. To determine whether GRIP1 could bind to a CAR-RXR-DNA complex, CAR-RXR, bound to biotinylated DNA containing four copies of the CYP2B1 NR1, was incubated with 35S-labeled GRIP1, and the complex was isolated by binding to streptavidin-agarose. Some nonspecific binding of 35S-GRIP1 to the streptavidin-agarose was observed if RXR was omitted from the reaction (Fig. 2, lane 2). However, specific binding of 35S-GRIP1 was observed as an increase in binding when both CAR and RXR were present (Fig. 2, compare lanes 2 and 4). Binding was further increased if the agonist TCPOBOP was added to the reaction (Fig. 2, lane 5), and the antagonist androstenol modestly reduced the binding (Fig. 2, lane 6), results that are consistent with the GST pull-down studies above. Approximately the same amount of CAR-RXR was bound to the biotinylated DNA in each reaction as shown by the Western blot of RXR bound to the DNA (Fig. 2, bottom panel).

The interaction of GRIP1 with CAR-RXR bound to DNA was also examined by gel mobility shift analysis. As shown previously (6, 11), CAR-RXR binds to the CYP2B1 NR1 (Fig. 3, lane 1). The addition of GST or GST-GRIP1-1 to reactions did not result in a supershift of the CAR-RXR complex (Fig. 3, lanes 2 and 3). In contrast, some radioactivity migrated more slowly than the CAR-RXR complex for GRIP1-2 and GRIP1-3, although not as discrete bands, suggesting that these fragments of GRIP1 interact with the CAR-RXR complex but that the binding is not stable under the conditions for gel electrophoresis. The addition of ligands had little effect on the binding of GST or GST-GRIP1-1 (Fig. 3, lanes 7, 8, 12, 13, 17, and 18). In contrast, TCPOBOP strongly increased the supershifted complex when GRIP1-2 and GRIP1-3 were added to the reactions (Fig. 3, lanes 9 and 10), whereas in the presence of androstenol, no supershift of the CAR-RXR complex was observed (Fig. 3, lanes 14 and 15). The reason for the difference in the mobility of the supershifted complexes for GRIP1-2 and GRIP1-3 is not known. These results are consistent with the GST pull-down and DNA affinity studies above, indicating that TCPOBOP increases and androstenol decreases the binding of GRIP1 to CAR and that the dependence of the binding on ligands requires the C-terminal sequence. Even though the strongest binding to CAR alone in the GST pull-down experiments was
Binding of $^{35}$S-GRIP1 to CAR-RXR-DNA complexes. CAR alone or RXR and CAR were incubated with a biotinylated DNA fragment containing four copies of the CYP2B1 NR-1-binding site for CAR-RXR. The complex of proteins with the DNA was purified by binding to streptavidin-agarose and was incubated with full-length $^{35}$S-GRIP1 (5–1121) synthesized by transcription/translation in the presence of absence of TCPOBOP (T) or androstenol (A) as indicated. The radioactive proteins bound to the streptavidin-agarose complex were eluted with 0.2% sarkosyl and analyzed by SDS-PAGE and autoradiography. The expected position for GRIP1 is indicated. At the bottom, a Western blot of the proteins in the streptavidin-agarose complex detected with antiserum to RXR demonstrated that similar amounts of CAR-RXR were binding to the DNA for each sample. These results were consistently observed in three independent experiments.

**Fig. 2.** Binding of $^{35}$S-GRIP1 to CAR-RXR-DNA complexes. CAR alone or RXR and CAR were incubated with a biotinylated DNA fragment containing four copies of the CYP2B1 NR-1-binding site for CAR-RXR. The complex of proteins with the DNA was purified by binding to streptavidin-agarose and was incubated with full-length $^{35}$S-GRIP1 (5–1121) synthesized by transcription/translation in the presence of absence of TCPOBOP (T) or androstenol (A) as indicated. The radioactive proteins bound to the streptavidin-agarose complex were eluted with 0.2% sarkosyl and analyzed by SDS-PAGE and autoradiography. The expected position for GRIP1 is indicated. At the bottom, a Western blot of the proteins in the streptavidin-agarose complex detected with antiserum to RXR demonstrated that similar amounts of CAR-RXR were binding to the DNA for each sample. These results were consistently observed in three independent experiments.

**Fig. 3.** Interaction of GRIP with CAR-RXR detected by gel mobility supershifts. A $^{32}$P-labeled oligonucleotide containing a CYP2B1 NR-1 site was incubated with CAR-RXR and either GST or fragments of GRIP1 fused to GST. An agonist, TCPOBOP, or an antagonist, androstenol (ANDRO), for CAR or an agonist for RXR, 9-cis-retinoic acid (9-cis-RA), was added as indicated. The samples were separated by nondenaturing gel electrophoresis, and radioactivity was detected by autoradiography. The position of CAR-RXR complexed to DNA is indicated, as is the region of supershifts containing GRIP1 fragments bound to the CAR-RXR complexes. These results were reproducibly observed in three independent experiments.

observed with the GRIP-1 fragment, little or no binding with GRIP-1 was detected in these gel shift studies. This suggests that the C-terminal region is required for stable binding of GRIP1 to CAR-RXR-DNA complexes but not for stable binding to CAR alone. Further, the influence of the C-terminal region of GRIP1 on binding is modulated by ligand binding.

The modulation of the interaction of GRIP1 with CAR-RXR by ligands raised the question of whether the RXR ligand 9-cis-retinoic acid would have any effect on the interaction. Interestingly, addition of cis-retinoic acid to the binding reaction resulted in increases in GRIP1 binding to CAR-RXR analogous to that observed with TCPOBOP (Fig. 3, lanes 17–20). Similar results have been observed with thyroid hormone receptor-RXR heterodimers (22). These results suggest that conformational changes induced in one nuclear receptor subunit of the CAR-RXR heterodimer can alter the conformation of the partner nuclear receptor and affect its interaction with a coactivator.

**Fig. 3.** Interaction of GRIP with CAR-RXR detected by gel mobility supershifts. A $^{32}$P-labeled oligonucleotide containing a CYP2B1 NR-1 site was incubated with CAR-RXR and either GST or fragments of GRIP1 fused to GST. An agonist, TCPOBOP, or an antagonist, androstenol (ANDRO), for CAR or an agonist for RXR, 9-cis-retinoic acid (9-cis-RA), was added as indicated. The samples were separated by nondenaturing gel electrophoresis, and radioactivity was detected by autoradiography. The position of CAR-RXR complexed to DNA is indicated, as is the region of supershifts containing GRIP1 fragments bound to the CAR-RXR complexes. These results were reproducibly observed in three independent experiments.

**GRIP1 Enhances CAR-mediated Transactivation in Cultured Cells**—To examine the functional consequences of the CAR and GRIP1 interactions, the effects of cotransfection of GRIP1 with CAR on transactivation of an enhancer with four copies of the CYP2B1 NR1 site fused to the CYP2C1 proximal promoter and a luciferase reporter were examined. In two hepatic cell lines, CAR transactivation was enhanced by expression of GRIP1.

Transfection of 1 ng of CAR expression vector alone resulted in about a 2-fold increase in luciferase activity in HepG2 cells, and coexpression of GRIP1 resulted in an additional 2-fold increase (not shown). In Hepa1c1c7 cells, 1 ng of CAR increased luciferase activity about 3-fold, and a dose-dependent enhancement of the CAR activation by GRIP-1 was observed (Fig. 4). Androstenol nearly abolished the effect of exogenous CAR expression in the absence of exogenous GRIP1, but this inhibition was partially reversed by expression of GRIP, suggesting that the androstenol decreased the interaction between CAR and GRIP (Fig. 4). TCPOBOP increased CAR-mediated transactivation by an additional 2-fold, and GRIP1 increased the transactivation further, but the fold-induction was less than in the untreated cultures (Fig. 4). This result is consistent with an increased affinity of GRIP1 for CAR as a result of TCPOBOP binding so that increased activation is observed with the lower concentrations of endogenous GRIP. These ligand effects on transcription are thus consistent with the modulation of in vitro binding of CAR and GRIP by ligands.

**Exogenous Expression of CAR in Hepatocytes in Situ Has Little or No Effect on Transactivation of the CYP2B1 PBRU**—The previous experiments examined CAR-mediated transactivation and the influence of GRIP1 in cultured cells. In vitro studies and disruption of the CAR gene in transgenic mice have demonstrated that CAR is the primary factor for PB induction of Cyp2b genes in mice (8). Although CAR transactivation in continuously cultured cells was used above as a model for the induction of CYP2B genes by PB, the PB response is not seen in these cells, and therefore the observed transactivation by CAR may not accurately reflect the induction of the genes in vivo. To examine the effects of expression of exogenous CAR and GRIP1 in vivo, transactivation of the PBRU was studied by transfection of mouse hepatocytes in vivo by injection of DNA into the...
tail vein. Among organs taking up the DNA, highest expression is observed in liver, and between 5 and 25% of hepatocytes are transfected (23). PB treatment has been shown to result in a >100-fold increase in expression of luciferase from a vector in which the PBRU of CYP2B1 and a minimal CYP2C1 promoter is fused to the luciferase gene using this method.5

In untreated animals, the expression of exogenous CAR in hepatocytes increased transactivation of the PBRU by about 3-fold (Fig. 5, inset). Because CAR is predominantly a cytoplasmic protein in untreated animals (11), the small increase indicates that overexpression of CAR in the hepatocytes results in some leakage into the nucleus. In PB-treated animals, the expression of luciferase is increased about 100-fold. If CAR is expressed exogenously in these PB-treated animals, there is essentially no additional increase. The lack of effect suggests that a component of the transactivation mechanism other than CAR is limiting in PB-treated animals.

Expression of GRIP1 in Vivo Synergistically Activates Transcription with CAR and Enhances the PB Response—To examine the effect of GRIP1 on PBRU transactivation, liver cells were transfected in situ by the tail vein injection procedure with expression vectors for GRIP1 and/or CAR. In the untreated animal, CAR is primarily cytoplasmic so the overexpression of GRIP1 would be expected to have little effect on transactivation of the PBRU. Surprisingly, expression of GRIP1 in hepatocytes in vivo increased luciferase expression about 15-fold in untreated animals, much more than expression of CAR alone did (Fig. 5, inset). Even more remarkably, coexpression of CAR with GRIP1 resulted in a synergistic increase of 150-fold in transactivation of the PBRU, which is similar to the induction observed with PB treatment. Although expression of exogenous CAR had little effect in PB-treated mice, expression of GRIP1 resulted in a 2-fold increase. CAR and GRIP1 together were modestly synergistic in PB-treated animals compared with untreated animals and resulted in a 4-fold increase (nearly 500-fold compared with untreated animals). The 2-fold increase observed with expression of CAR in

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**Fig. 4.** Enhancement of CAR-mediated transactivation by GRIP1 in Hepa1c1c7 cells and effect of ligands. Hepa1c1c7 cells were transfected with a firefly luciferase reporter, which contained four copies of the CYP2B1 NR-1 site fused to the CYP2C1 proximal promoter, an SV40 promoter/Renilla luciferase reporter as an internal standard, and with expression vectors for CAR and GRIP1, as indicated. The values for firefly luciferase were normalized by dividing by the Renilla luciferase values. Me2SO (−), 50 µM androstenol (ANDRO), or 5 µM TCPOBOP were added to the cultures as indicated. The standard errors of the mean are indicated for six transfected wells from two independent experiments.

**Fig. 5.** Transactivation by CAR and GRIP1 in murine hepatocytes transfected in situ. DNA of expression vectors for CAR and/or GRIP1, a firefly luciferase reporter with the CYP2B1 PBRU fused to the CYP2C1 promoter, and a Renilla luciferase reporter containing the SV40 enhancer/promoter were injected into mouse tail veins as described under "Materials and Methods." 6 h after the DNA injections the mice were injected intraperitoneally with either saline or 100 mg/kg body weight PB. 24 h after injection, the mice were sacrificed, and the luciferase activities in liver extracts were determined. The firefly luciferase activities were normalized by dividing by the Renilla luciferase activities. The inset shows the first three bars with the scale on the abscissa expanded 10X. The standard errors of the mean are indicated for four to eight independent determinations.
cence, but the relative nuclear localization was greater in the PB-treated groups than in the untreated (CONTROL) group in which exogenous GRIP1 was expressed (Fig. 6). To provide a semiquantitative estimate of nuclear localization, the relative intensity of fluorescence in the nucleus was quantified, and a histogram of the nuclear intensity in 20 cells in each group was plotted (Fig. 7). A value of 0 or 1 indicates 100% cytoplasmic or 100% nuclear localization, respectively. In untreated animals, 75% of cells had less than 0.25 relative nuclear fluorescence (Fig. 7). In contrast, in PB-treated animals, 75% of cells had greater than 0.75 relative nuclear fluorescence, and all cells had greater than 0.25 nuclear fluorescence, which is consistent with earlier studies showing nuclear localization after PB treatment (11, 24). The expression of GRIP1 in untreated animals resulted in nuclear fluorescence intermediate between these two groups, but all cells had relative nuclear intensities greater than 0.25, and 40% of the cells had relative intensities greater than 0.75 (Fig. 7). GRIP1 expression did not detectably change localization of CAR in the PB-treated mice. These results show that exogenous expression of GRIP1 causes nuclear translocation of CAR in hepatocytes in vivo in the absence of ligand.

**DISCUSSION**

Previous studies have implicated p160 coactivators in the transactivation mediated by CAR, but these studies rested on showing interactions of SRC-1 with CAR alone (10, 12) and modest increases in CAR transactivation resulting from overexpression of SRC-1 in cultured cells (7, 24). The present studies confirm the interaction of CAR with a p160 coactivator, GRIP1, and further show that GRIP1 interacts with CAR/HRXR heterodimers bound to DNA, which is a more functional form of these nuclear receptors. CAR-mediated transactivation of either the PBRU (data not shown) or four copies of the CYP2B1 NR-1 was enhanced 2–3-fold by expression of GRIP1 in cultured cells. These results are similar to those obtained for the PBRU or βRARE sites when SRC-1 was coexpressed with CAR in primary cultures of hepatocytes or CV-1 cells, respectively (7, 10), although transactivation of 2 NR-1 sites was not increased by SRC-1 in primary hepatocytes in contrast to the present results with four copies of the NR-1. Although CAR transactivation in cultured cells was only modestly enhanced by GRIP1, exogenous expression of GRIP1 in vivo in untreated mice increased transactivation by 15-fold and increased transactivation by 50-fold in cells exogenously expressing CAR. In PB-treated animals, exogenous expression of GRIP1 enhanced CAR transactivation about 2-fold. The dramatic 50-fold increase in CAR transactivation mediated by GRIP1 expression in vivo in the untreated animals, compared with 2–3-fold in-
creases in cell culture, provides strong additional evidence for the role of p160 coactivators in PB induction of CYP2B genes. GRIP1 has been shown to activate hepatic nuclear receptors, and either the protein or mRNA was reported to be present in human or mouse liver (25–27), but it was recently reported that GRIP1 was not detectable in hepatic parenchymal cells using immunocytochemical techniques (28). Thus, either SRC-1 or AI1p/pCIP/3SRC-3, the latter of which is predominantly expressed in Xenopus liver (29), may function as the p160 form interacting with CAR in the liver.

The binding of GRIP1 to GST-CAR in vitro was increased by the agonist TCPBOB and decreased by the antagonist androstenol, which is consistent with earlier studies in which ligands modulated binding of SRC-1 to CAR (10, 12). Interesting differences were observed in the binding between CAR and different fragments of GRIP1 containing the central receptor interacting sites and either the N-terminal portion or the C-terminal portion of molecule. Both types of fragments bound to CAR alone, but binding was modulated by ligand only for the C-terminal fragment. In gel shift assays, little binding of GRIP1 to CAR-RXR DNA complexes was observed with the N-terminal fragment, but binding was observed with the C-terminal fragment even if only one of the three LXXLL motifs in the nuclear interaction domain was present. This binding was increased by the CAR agonist TCPBOB and decreased by the antagonist androstenol. The role of LXXLL in binding of GRIP1 to CAR has not been established, but there appears to be little specificity for individual LXXLL motifs because mutation individually of each of the three LXXLL motifs in the nuclear receptor interaction domain did not alter the interaction of CAR and the related p160 coactivator from Xenopus, xSRC-3 (29). These results suggest that effects on the binding of GRIP1 to CAR by ligands is mediated through the C-terminal portion of the molecule, analogous to ligand-dependent nuclear receptors, such as the steroid hormone receptors, and that the C-terminal portion is required for stable binding of GRIP1 to CAR under the conditions of the gel shift assay.

It has been reported that exogenous expression of CAR inhibited PB induction of CYP2B1 in primary cultures of hepatocytes and that changing the PBRU NR-1 site to a βRARE site, which still binds CAR, eliminated PB induction (30). These results led to the proposal that CAR was not involved in PB induction of CYP2B genes. An alternate explanation of these results is that CAR is saturating in the nucleus after PB treatment, so that expression of additional CAR has little effect and may be inhibitory. The present result that exogenous expression of CAR in PB-treated animals does not increase transactivation unless GRIP1 is expressed exogenously as well provides support for this alternate explanation and suggests that the p160 coactivator is limiting relative to CAR in the PB-treated hepatocyte. The loss of PB induction in transgenic mice with disrupted CAR genes (8) and other studies showing that exogenous CAR expression increased transactivation of the PBRU in PB-treated primary cultures of hepatocytes or hepatocytes transfected in situ by bolistic particles (7) also support a role for CAR in PB induction. Possible explanations for the loss of PB induction observed with the conversion the PBRU NR-1 site to a βRARE site (30) are that the conformation of CAR-RXR may be different when bound to the βRARE site (31) or the alignment of CAR-RXR with other proteins binding to the PBRU may be changed (32), resulting in a loss of transactivation.

The most surprising result in this study was the dramatic synergistic effects of exogenous CAR and GRIP1 expression in untreated animals resulting in a 150-fold increase in transactivation. The basis for the synergistic effect is most likely due to two effects of the expression of exogenous GRIP1, translocation of CAR to the nucleus, and direct activation of CAR by GRIP1. Exogenous expression of GRIP1 resulted in nuclear localization of GFP-CAR in essentially all of the transfected hepatocytes, although the extent of translocation was heterogeneous and less complete than that observed in PB-treated cells. Direct activation of CAR by GRIP1 is about 2-fold based on the effects of GRIP1 in cultured cells and in PB-treated mice, so that most of the synergistic 150-fold effect in untreated animals is the result of translocation ofCAR to the nucleus. Because in cultured cells, CAR is always present in the nucleus, the difference in localization of CAR explains most of the dramatic difference in magnitude of the GRIP1 effect in cultured cells and in vivo. In addition, the PBRU is a complex enhancer with DNA-binding proteins other than CAR contributing to the PB response, (4, 15–17) and coactivators or cosuppressors other than the p160 coactivators may be also recruited to the PBRU. The differences in concentration of these factors in cultured cells and in vivo may contribute to the differences observed for the effects of GRIP1 expression in these two systems.

These studies raise the possibility that PB activation of GRIP1 might contribute to the translocation of CAR to the nucleus after PB treatment. Although the action of PB is poorly understood, the phosphatase inhibitor, okadaic acid, inhibits CAR nuclear translocation (11). The target of the phosphatase, presumed to be CAR, has not been directly identified. Precedents exist for modulation of GRIP1 activity by phosphorylation (33) and for translocation of regulatory proteins by coregulators, for example translocation of histone deacetylase-4 by SMRT (silencing mediator for retinoic acid receptor and thyroid hormone receptor) (34), so that it is possible that dephosphorylation of a p160 coactivator could contribute to the PB response. An activated GRIP1 could mediate translocation of CAR either by interacting with cytoplasmic CAR and inducing nuclear translocation or by interacting with nuclear CAR and enhancing of nuclear retention of CAR. The latter mechanism would be possible if CAR is continuously shuttling between the nucleus and cytoplasm even in untreated animals as has been proposed for nuclear receptors (35). Any GRIP effect on CAR nuclear translocation would have to be independent of its effect on activation of CAR because CAR with the C-terminal transactivation domain inactivated by deletion is still translocated to the nucleus (24). Further studies will be required to establish the mechanism by which p160 coactivators induce CAR nuclear translocation and the role of this effect in PB induction of CYP genes.

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