Structural Determinants of Constitutive Androstane Receptor Required for Its Glucocorticoid Receptor Interacting Protein-1-mediated Nuclear Accumulation*

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Nuclear translocation of constitutive androstane receptor (CAR) is a primary mechanism for the induction of cytochrome P450 genes by phenobarbital (PB). We have shown that exogenous expression of the p160 coactivator glucocorticoid receptor interacting protein-1 (GRIP1) in hepatocytes in vivo can mediate PB-independent nuclear accumulation of murine CAR (mCAR). To understand the mechanism of this PB-independent nuclear accumulation, we have examined the mCAR structural determinants of its GRIP1-mediated nuclear localization. Mutations of the xenobiotic response sequence (XRS), which had been shown to block PB-dependent nuclear translocation of human CAR in mouse hepatocytes in vivo, also blocked GRIP1-mediated nuclear accumulation of mCAR in mouse hepatocytes in vivo and further blocked nuclear localization in cultured HepG2 cells. A leucine 326 XRS mutant retained partial transcriptional activity, but mutations of three leucines in the XRS eliminated transcriptional activity in HepG2 cells, suggesting that the translocation function of the XRS overlaps with transcriptional functions. Mutation of the activation function 2 motif, by deletion of the C-terminal 8 amino acids, also reduced nuclear localization by both PB treatment and GRIP1 expression in hepatocytes in vivo, suggesting that either interaction with GRIP1 through this motif or active CAR was required for the nuclear localization. The localization of a DNA-binding domain mutant was essentially unchanged by coexpression of GRIP1, although without GRIP1 coexpression, this mutant expressed a more nuclear localization compared with wild type. The results are most consistent with a model in which GRIP1 interaction and activation of mCAR in the nucleus result in retention and accumulation of mCAR in the nucleus in untreated animals. The model requires that mCAR is constantly shuttling between the nucleus and cytoplasm even in untreated animals in which mCAR is predominantly cytoplasmic.

The cytochrome P450 superfamily is composed of a group of monooxygenases that metabolize diverse exogenous compounds such as drugs and toxic agents, as well as endogenous compounds like steroids, fatty acids, and prostaglandins (1). The activities of subsets of cytochrome P450s are induced by xenobiotics. Phenobarbital (PB) is the prototype of a group of structurally diverse xenobiotic chemicals that induce several subfamilies of cytochrome P450 genes, including CYP2B, CYP2C, and CYP3A, of which the most dramatic effects are observed on the CYP2B subfamily (2). In addition, PB also induces the expression of other xenobiotic-metabolizing enzymes such as glutathione S-transferases (GST) and UDP-glucuronosyltransferases.

PB induction of CYP2B genes has been shown to be mediated by the nuclear receptor constitutive androstane receptor (CAR) (3, 4). CAR is unusual among nuclear receptors in having a high constitutive activity when assayed in cultured cells (5). The initial ligands identified for CAR were androstanes that were reverse agonists and down-regulated CAR activity (6). Later a subset of PB-like inducers typified by 1,4-bis[3,5-dichloropyridyloxy]benzene (TCPOBOP) were found to be agonists that enhanced CAR-mediated transactivation in HepG2 cells and potentiated binding of p160 coactivators to CAR (7). The cellular location of CAR is key to its role in regulating CYP2B genes in response to PB treatment. In untreated animals, CAR is predominantly present in the cytoplasm, and PB treatment results in the translocation of CAR into the nucleus (8–10). In the nucleus, CAR forms a heterodimer with retinoid X receptor and binds to direct repeat-4 nuclear receptor-binding sites in a complex PB-inducible enhancer, termed the PB-responsive unit (PBRU), which leads to activation of gene expression (3, 11). Interestingly, in continuously cultured cells like HepG2 cells, CAR is located in the nucleus and is constitutively active so that PB induction cannot be studied in these cells (8).

Nuclear accumulation of CAR is the first step in CAR activation and thus plays an essential role in CAR-mediated gene transcription. Protein trafficking between the cytoplasm and nucleus is mediated by nuclear localization signals (NLS) for nuclear import and nuclear export signals for nuclear export, which interact with specific transport receptors (12). Classical NLS or nuclear export signal motifs are not present in CAR. However, mutational analysis of human CAR (hCAR) identified a leucine-rich sequence in the C-terminal region of the protein, termed the xenobiotic response sequence (XRS), which

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¶ The abbreviations used are: PB, phenobarbital; CAR, constitutive androstane receptor; mCAR, murine CAR; hCAR, human CAR; PBRU, PB-responsive unit; NLS, nuclear localization signals, DBD, DNA-binding domain; AF, activation function; XRS, xenobiotic response sequence; FP2A, protein phosphatase 2A; CRIP, CAR cytoplasmic retention protein; GRIP1, glucocorticoid receptor interacting protein-1; GFP, green fluorescent protein; GST, glutathione S-transferase; TCPOBOP, 1,4-bis[3,5-dichloropyridyloxy]benzene; RNF, relative nuclear fluorescence.

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was required for the PB-induced nuclear translocation of hCAR expressed in mouse liver in vivo (10). The XRS motif resembles nuclear export signal motifs present in the aryl hydrocarbon receptor and human immunodeficiency virus type 1 Rev rather than typical NLS motifs (13, 14). It has been proposed that interaction of the XRS with another unknown protein is responsible for its nuclear localization function (10). Deletion of the N-terminal portion of hCAR, including the DBD, or mutation of the activation function-2 (AF-2) domain did not affect PB-mediated nuclear translocation of hCAR in these studies. The protein phosphatase inhibitor okadaic acid, which inhibits protein phosphatase 2A (PP2A), can also inhibit PB-induced nuclear accumulation of CAR (8). PP2A was recruited to a cytoplasmic complex of CAR and heat shock protein 90 in mouse hepatocytes after PB treatment (15). In addition, interaction of a protein, CAR cytoplasmic retention protein (CCRP), with CAR was detected by yeast two-hybrid analyses, and overexpression of CCRP increased the relative amounts of CAR in the cytoplasm in HepG2 cells (16). It was proposed that CCRP retains CAR in the cytoplasm and that the recruitment of PP2A and the resulting dephosphorylation release CAR from the heat shock protein 90-CCRP complex and facilitate its translocation into the nucleus. Whether CAR is the direct target for dephosphorylation by PP2A is not known.

Recently, we found that PB-independent localization of mCAR in the nucleus could be mediated by exogenous expression of the coactivator GRIP1 in hepatocytes in vivo (9). The increased nuclear localization of mCAR and the activation of mCAR by GRIP1 resulted in a dramatic synergistic increase in transcription of a PBNU reporter gene in livers of untreated mice treated with PB (15). The mechanism by which GRIP1 causes nuclear localization of mCAR is not known, nor is it known whether the mechanism is related to that of PB-mediated nuclear localization. In the present study, we show that mutation of the XRS blocks nuclear localization of mCAR by GRIP1 in hepatocytes in vivo and also blocks nuclear translocation in cultured cells. Deletion of the C-terminal 8 amino acids to inactivate the AF-2 domain reduces nuclear localization by both PB treatment and GRIP1 expression, whereas GRIP1 expression had little effect on the nuclear localization of a DBD mutant. The results are consistent with a model in which the interaction of GRIP1 with nuclear mCAR and the activation of mCAR by GRIP1 results in nuclear retention.

MATERIALS AND METHODS

Plasmids—The expression vectors pGEX2TKCAR, pGEX2TKCARΔS (CAR1–350), and pEGFPc1CAR have been described (9, 17). The mutations in the mCAR mutants, C21A/C24A, L326A, and L322A/L326A/L329A, were introduced by the QuikChange site-directed mutagenesis system in the pEGFP1CAR or pGEX2TKCAR vectors as described by the manufacturer (Stratagene, La Jolla, CA). For the deletion mutant CAR1–328, the sequence encoding amino acids 1–328 was amplified by PCR with pGEX2TKCAR plasmid DNA as template and primers that introduced BamHI and EcoRI sites at the 5′ and 3′ ends, respectively. The amplified fragment was digested with BamHI and EcoRI and inserted into the BamHI/EcoRI site of pGEX2TK or the BglII/EcoRI site of pEGFPc1. An NLS sequence was inserted into wild type, L326A, L322A/69A, and CAR1–328-GFP constructs at the N terminus of GFP using the QuikChange site-directed mutagenesis system and the mutagenic primer, 5′-GGTGCAGCAACATGCAAAAGAGAACCTAAAGCTCTGACAAGGAACG-3′. The inserted nucleotides, which encode the new NLS, are underlined. pGFPc1CAR1–350 was made by substituting the BamHI/EcoRI fragment from pGEX2TKCARΔS for the same fragment in pEGFPc1CAR. All of the mutations and deletions were confirmed by sequencing. The mammalian expression vector pSG5.HA-GRIP1 encoding full-length GRIP1 was obtained from M. R. Stulacup (18).

Transient Transfection Assay in Cultured Cells—Human HepG2 and monkey kidney Cos1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units/ml of penicillin at 37 °C under a 5% CO2 atmosphere. Transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Expression vectors were transfected into each well by transfection, 0.5 μg of pBRL-CYP2C1-Luc DNA (19), which contains the CYP2B1 PBRU fused to the minimal CYP2C1 promoter and the firefly luciferase reporter gene, 10 ng of pRL-SV40 DNA containing the SV40 promoter and Renilla luciferase gene, and various amounts of mCAR and GRIP1 expression vector DNA were added to each well. After 24 h, the cells were lysed, and the luciferase activities were determined by using dual luciferase reporter assay system (Promega Biotech), and the firefly luciferase value was normalized to the Renilla value for each sample.

Localization of GFPs in HepG2 Cells—HepG2 cells grown on coverslips (Corning) in a 6-well plate were transfected with the DNA of expression vectors for the fluorescent proteins with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After incubation for 24 h, the cells were fixed with 2% paraformaldehyde. The cells were then permeabilized by incubation for 5 min in 0.1% Triton X-100 in phosphate-buffered saline followed by incubation in 100 μg/ml DNase-free RNase in phosphate-buffered saline at 37 °C for 10 min. Finally, the cells were mounted with Vectashield mounting medium with propidium iodide (Vector Laboratories Inc., Burlingame, CA). Fluorescence was detected with a Zeiss LSM confocal microscope. The C-terminal XRS motif is identified by visualization of DNA by epifluorescence microscopy. The relative nuclear intensity of green fluorescence from the GFP was determined as described (9) by drawing a line across the center of cell and determining the fluorescence intensities, 1, or 1, for the nucleus or cytoplasm, respectively. Relative nuclear fluorescence (RFN) was calculated as 1/(1 + 1) so that a value of 1 is obtained when the fluorescence is 100% nuclear and a value of 0 is obtained when the fluorescence is 100% cytoplasmic.

Transient Transfection and Localization of mCAR in Vivo—Plasmid DNA for injection into tail veins of mice was 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 System (Mirus Bio Corp.) as described (9). For transient transfection transcriptional assay, 10 μg of pPBNU-CYP2C1-Luc DNA, 0.5 μg of pRL-SV40 DNA, and, as indicated, 1 or 10 μg of expression vector DNA for mCAR or GRIP1, respectively, were injected. 24 h after injection, the mice were sacrificed, the liver tissue was homogenized, and luciferase activity was determined by the dual luciferase assay as described (9).

For localization of wild type and mutant mCAR proteins, 5 μg of expression vectors for GFP-mCAR fusion proteins and 25 μg of GFP expression vector DNA were injected. Mice were treated intraperitoneally with isotonic saline or 100 μg/g of body weight of PB 2 h after injection of the DNA, and the animals were sacrificed 4 h later. The livers were cut into small pieces, placed in Tissue-Tek O.C.T. Compound (Miles, Inc.), and frozen in liquid N2. Frozen sections of 10 μm were prepared with a cryostat microtome. Fixation, staining of nuclear DNA with propidium iodide, and detection of fluorescence were performed as described (9).

GST Pull-down Assay—The GST fusion proteins were expressed in Escherichia coli BL21 and purified by binding to glutathione-Sepharose (Amersham Biosciences). 32P-Labeled proteins were synthesized by in vitro transcription and translation according to the manufacturer's instructions (Promega Corp.). One μg of GST or GST fusion protein was incubated with 3 μl of the lysate containing the labeled proteins in 100 μl of binding buffer (20 mM Hepes, pH 7.6, 0.1 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.05% Nonidet P-40 in the presence of protease inhibitors). In some binding reactions, 10 μM TDCBOP was added to the reactions. After the incubation, the Sepharose beads were extensively washed in binding buffer and resuspended in SDS-PAGE sample buffer. The samples were boiled at 100 °C for 5 min and separated by SDS-PAGE. Radioactive proteins were visualized by autoradiography.

RESULTS

GRIP1 Cannot Induce Nuclear Accumulation of mCAR Mutants in Mouse Liver in Vivo—The XRS motif is required for PB-dependent nuclear accumulation of hCAR (10). To examine whether the XRS motif is also required for GRIP1-mediated nuclear accumulation of mCAR, the mutations L326A and L322A/L326A/L329A within the XRS and CAR1–328, which deletes the AF-2 domain and Leu-329 in the XRS, were con-
interactions of the DBD, XRS, and AF-2 domain are indicated. Cysteines and leucines that were mutated in the DBD and XRS, respectively, are underlined and in italics in the sequences shown for these regions. The sequence of 6 amino acids deleted from the C terminus in the AF-2 region is also shown.

As expected, the interaction of mCAR with GRIP1 in GST pull-down assays was largely inhibited by the deletion of 30 C-terminal amino acids in CAR(1–328) (Fig. 2). Interactions of the L326A and L322A/L326A/L329A mutants with GRIP1 were detectable and only slightly reduced compared with wild type mCAR in incubations without TCPOBOP. Addition of TCPOBOP substantially increased the interaction of GRIP1 with wild type mCAR as expected but did not increase the interaction of GRIP1 with L322A/L326A/L329A and only slightly increased the interaction with L326A. These results suggest that GRIP1 can still bind to the L322A/L326A/L329A and L326A mutants but that these mutations inhibit either TCPOBOP binding or the activating conformational change that results from TCPOBOP binding.

Mouse hepatocytes were transfected in vivo with expression vectors for GFP-tagged wild type or mutant mCARs, and the cells were imaged by confocal microscopy. Examples of images of two cells that exhibit predominantly cytoplasmic or nuclear localization for each construction are shown in Fig. 3, and the distribution of relative nuclear fluorescence in 40 randomly selected cells is shown in Fig. 4. When exogenous GRIP1 was coexpressed with the mCAR chimera in hepatocytes in vivo, the distribution of wild type mCAR was shifted toward the nucleus as described previously (Fig. 4, A and B) (9). In contrast, coexpression of GRIP1 had little effect on the distribution of the XRS leucine mutants and CAR(1–328) (Fig. 4, C–H), which indicates that the XRS is required for GRIP1-mediated nuclear localization of mCAR. Consistent with previous studies (10), these mutants also blocked the PB-dependent nuclear translocation of mCAR. The loss of both GRIP1- and PB-mediated nuclear accumulation of XRS mutant mCARs indicates that the XRS sequence is indispensable for nuclear translocation of mCAR.

The AF-2 Domain Is Also Involved in Both GRIP1-mediated and PB-induced Nuclear Accumulation of mCAR—AF-2 domains at the C-terminal ends of nuclear receptors are critical for the interaction of the receptors with coactivators. Mutation of critical amino acids within the AF-2 of CAR has been shown to eliminate the binding of CAR to p160 coactivators (20). To determine whether interaction of GRIP1 with the AF-2 of mCAR is critical for its GRIP1-mediated nuclear accumulation, we constructed a mutant, CAR(1–350), in which the C-terminal 8 amino acids, which are part of the AF-2 domain of mCAR but not the XRS motif, were deleted. Interaction of this mutant with GRIP1 was largely eliminated as expected (Fig. 2). Coexpression of GRIP1 did not affect the distribution of CAR(1–350) (Fig. 4, I and J) in untreated or PB-treated mice, indicating that the interaction of the AF-2 domain of mCAR with GRIP1 or a transcriptionally active mCAR is required for the nuclear accumulation.

Surprisingly only a very modest nuclear accumulation of the CAR(1–350) mutant was observed after PB treatment, much less than that observed with wild type mCAR (Fig. 4J). Cells with fluorescence predominantly in the nucleus (RNF > 0.5) increased slightly from 15 to 20% after PB treatment with the CAR(1–350) mutant, compared with a substantial increase from 5 to 63% with wild type mCAR. This result suggests that an intact C-terminal AF-2 domain contributes to PB-responsive nuclear accumulation of mCAR in contrast to a previous report that mutation of the AF-2 in hCAR did not affect PB-mediated nuclear translocation (10).

The mCAR DNA-binding Domain Is Required for GRIP1-mediated Nuclear Localization of mCAR—The mutation of the AF-2 domain eliminates both the activity of mCAR and the interaction of GRIP1 with CAR. To try to determine which of these two effects was required for the loss of GRIP1-mediated nuclear localization, the activity of mCAR was eliminated by mutating two Cys residues in the DBD of mCAR to Ala. These mutations, which block DNA binding, only slightly reduced the interaction of GRIP1 with mCAR (Fig. 2). In the absence of GRIP1 expression, this mutation resulted in a shift to a more nuclear localization (compare open bars in Fig. 4, A and K). Although the expression of GRIP1 resulted in a substantial shift of localization of the GFP-CAR proteins for wild type (Fig. 4A), expression of GRIP1 has a modest effect, if any, on the localization of the DBD mutant (Fig. 4K). These results suggest that GRIP1-mediated translocation is reduced in the DBD mutant, but this interpretation is complicated by the change in distribution of the DBD mutant alone, which might mask an effect of GRIP1. PB-mediated nuclear translocation was not greatly affected by the DBD mutant (Fig. 4, K and L). The absence of an effect of GRIP1 on the localization of the DBD mutant would be consistent with the hypothesis that the activity of mCAR contributes to nuclear localization because the DBD mutant is inactive but still interacts with GRIP1.
results. These results suggest that the function of the XRS motif is associated with basic nuclear import machinery so that mutation of XRS blocks transport into the nucleus in both cultured cells and hepatocytes in vivo.

The role of the AF-2 motif in cellular localization in HepG2 cells was also examined (Fig. 5E). The GFP-CAR(1–350) mutant displayed a pattern between those of wild type and XRS mutant mCARs, with 70% of cells, rather than 97.5% for wild type, having an RNF of >0.5. Expression of exogenous GRIP1 did not substantially change the distribution of the CAR(1–350) mutant, which is consistent with the studies in vivo. The slightly increased cytoplasmic localization of the CAR(1–350) mutant is consistent with the idea that active mCAR is retained in the nucleus, but changes in mCAR conformation that reduce cytoplasmic retention cannot be eliminated by these data.

Mutations of the DBD in CAR(C21A/C24A) did not greatly affect the distribution of mCAR in HepG2 cells (Fig. 5F). If anything, there was a slight increase in nuclear localization consistent with increased nuclear localization of this mutant in hepatocytes in vivo in untreated mice.

Effects of XRS Mutants on Transactivation of the PBRU in HepG2 Cells—The CAR XRS motif overlaps with the ligand binding and dimerization domains and is near the AF-2 domain (10, 21), so that the activity of CAR as well as its cellular location could be affected by XRS mutations. Transactivation of the PBRU fused to the CYP2C1 promoter by mCAR mutants was examined in HepG2 and COS1 cells transfected with an expression vector for wild type or mutant mCARs with or without an expression vector for GRIP1. Transfection of wild type mCAR alone increased reporter luciferase activity about 2-fold in HepG2 cells and had little effect in Cos1 cells (Fig. 6). Expression of GRIP1 alone had little effect in either cell type (Fig. 6). Coexpression of GRIP1 with wild type mCAR further increased the reporter activity by 2–3-fold in HepG2 cells, consistent with previous studies (9), and 9-fold in Cos1 cells compared with mCAR alone (Fig. 6). In contrast, when the XRS leucine mutants and CAR(1–328) were transfected alone, no significant increase in transactivation was detected. Coexpression of GRIP1 did not increase the transactivation activity of the L322A/L326A/L329A or CAR(1–328) mutants, but 2- and 5-fold increases were observed for the L326A mutant in HepG2 and Cos1 cells, respectively (Fig. 6).

Because the XRS mutants are localized primarily in the cytoplasm rather than the nucleus (Fig. 5), decreased activity of these mutants could be due to the decreased concentrations in the nucleus. To examine this possibility, NLS sequences were fused to the N-terminal side of the GFP-CAR chimera for the L326A, L322A/L326A/L329A, and 1–328 mutants. The presence of the NLS converted the distribution from primarily cytoplasmic to primarily nuclear for each mutant (Fig. 7). In wild type CAR, the addition of the NLS sequence did not decrease transcriptional activity. For the mutants, the pattern of transcription and response to exogenous GRIP1 was similar to that of the mutants without the NLS sequence (compare Fig. 6 with Fig. 8). The L322A/L326A/L329A and 1–328 mutants were inac-
nuclear part (through the center of the nucleus. The fluorescent intensity of the fluorescence was calculated by drawing a line across a confocal plane randomly selected for each mCAR construction, and the relative nuclear intensities of the nuclear and cytoplasmic parts (C21A/C24A).

**Fig. 3.** Cells were transfected with expression vectors for WT GFP-CAR imaging, and treatment of animals were as described in the legend to mCAR chimera in hepatocytes

**Fig. 4.** Cellular localization of wild type and mutant GFP-mCAR chimera in hepatocytes in vivo. Transfection of hepatocytes, imaging, and treatment of animals were as described in the legend to Fig. 3. Cells were transfected with expression vectors for WT GFP-CAR (A, B) or for GFP-CAR mutants, L326A (C, D), L322/6/9/A (E, F), CAR(1–328) (G, H), CAR(1–350) (I, J), or DBM (K, L). 40 cells were randomly selected for each mCAR construction, and the relative nuclear fluorescence was calculated by drawing a line across a confocal plane through the center of the nucleus. The fluorescent intensity of the nuclear part ($I_n$) of the line was divided by the sum of the fluorescent intensities of the nuclear and cytoplasmic parts ($I_n + I_c$) of the line to calculate the relative nuclear florescence. WT, wild type; DBM, C21A/C24A.

**UNtreated vs. PB**

**Relative Nuclear Fluorescence [ln(ln+1c)]**

In the CAR(1–328) mutant, the AF-2 domain, as well as Leu-329 in the XRS motif, is deleted so that no activity would be expected. On the other hand the AF-2 is intact in the inactive L322A/L326A/L329A mutant, which suggests that the XRS nuclear translocation activity overlaps with transactivation functions of mCAR.

**Effect of XRS Mutants on Transactivation of the PBRU by mCAR in Hepatocytes in Vivo**—We have shown that coexpression of wild type mCAR with GRIP1 results in a dramatic synergistic activation of a PBRU reporter gene in untreated animals (9). Because cultured cells may have different concentrations of regulatory factors than cells in vivo, which may influence the results, we also examined the effects of mutations in the XRS and AF-2 domains on transactivation of the PBRU in hepatocytes in vivo in untreated mice. As observed previously (9), transfection of mCAR alone resulted in a modest increase in transactivation of the PBRU, whereas transfection of GRIP1 resulted in a more substantial increase, and a strong synergistic response was observed when both mCAR and GRIP1 were expressed (Fig. 9). Expression of the L326A, L322A/L326A/L329A, and CAR(1–328) mutants did not result in significantly increased activation of the PBRU, nor was there any synergistic increase when expressed with GRIP1 (Fig. 9). Although there is a slight increase in activity of the L326A mutant when GRIP1 is coexpressed (Fig. 9), this increase was the result of a single high value for 1 of the 5 animals in the group, which is reflected in the large standard error so that the increase is not significant. Although L326A was predominantly located in the cytoplasm in cells coexpressing GRIP1, about 40% of the cells had RNfs of 0.25 or greater, suggesting that a significant amount of CAR was in the nuclei of these cells (Fig. 4C). Because this mutant was transcriptionally active in HepG2 cells when GRIP1 was coexpressed, the lack of activity in the hepatocytes in vivo suggests that the nuclear fluorescence in these cells represents an inactive form of the GFP-mCAR fusion protein.

Unexpectedly, transactivation after expression of the CAR(1–350) mutant was less than the activity from endogenous mCAR and GRIP1 in cells not transfected with the expression vectors (Fig. 9). These results suggest that the CAR(1–350) mutant inhibited the activity of the endogenous mCAR or GRIP1, but we were unable to demonstrate a dominant negative effect in cultured cells (data not shown). The reason for the decrease is not known.

**DISCUSSION**

CAR is primarily located in the cytoplasm of hepatocytes in untreated animals and accumulates inside the nucleus after PB treatment. Although the details of the regulatory mechanisms involved in cellular localization of CAR are not known, it is generally thought that CAR is retained in the cytoplasm as a complex that is then translocated into the nucleus after PB treatment (15). It was then surprising that exogenous expression of the coactivator GRIP1 in hepatocytes in vivo resulted in the PB-independent nuclear localization of mCAR in untreated animals (9). There are two basic ways in which GRIP1 could cause the nuclear localization of mCAR. The first would involve an interaction of GRIP1 with the cytoplasmic complex containing mCAR that would result in release from cytoplasmic retention and transport into the nucleus. The second would involve an interaction of GRIP1 with mCAR in the nucleus that would stabilize mCAR in the nucleus or prevent its nuclear export. To distinguish between these possibilities, sequences in mCAR were mutated that are required for PB-induced nuclear translocation, that affect the interaction of GRIP1 with mCAR, and that affect activity but not interaction with GRIP1. In each case mutation of the relevant mCAR sequences resulted in at least a partial loss of GRIP1-mediated localization of mCAR in the nucleus. In the case of the DBD mutation, the interaction with...
GRIP1 was not greatly affected, but this mutation blocks DNA binding and transactivation by mCAR. Expression of GRIP1 caused little change in the distribution of the DBD mutant, whereas GRIP1 expression substantially changes the distribution of wild type CAR. This results suggest that GRIP1-mediated nuclear accumulation of CAR is reduced for the DBD mutant, but this mutation alone results in a more nuclear distribution, which might mask a GRIP1 effect. Nevertheless, the loss of GRIP1-mediated changes in localization is consistent with the hypothesis that the GRIP1 effect is related to the activation of mCAR by GRIP1 in the nucleus and not to a cytoplasmic interaction that results in nuclear translocation. Consistent with this conclusion, predominant nuclear localization of GRIP1 has been observed in most studies (22, 23).

This conclusion requires that even in the untreated animal, mCAR is not simply sequestered in the cytoplasm but is constantly shuttling between the nucleus and the cytoplasm with the steady state levels predominantly cytoplasmic in the untreated animal. This is consistent with the cellular trafficking of many nuclear receptors that are constantly shuttling between the nucleus and cytoplasm whether activated or not (24). The interaction of exogenously expressed GRIP1 with mCAR, resulting in increased activation of the small pool of nuclear mCAR in the untreated animal, then could result in reduced export from the nucleus and increased nuclear localization. Alternatively, the activated GRIP1-CAR complex could reduce nuclear degradation of unactivated CAR. Although such a mechanism for coactivator modulation of nuclear receptor cellular localization has not been described, the expression of another p160 coactivator, SRC1, has been shown to reduce the mobility of the estrogen receptor in the nucleus (24). Such reduced mobility would be consistent with sequestration in the nucleus and increased nuclear localization of mCAR.

Whether GRIP1-mediated nuclear accumulation of mCAR is related to the physiological activation of mCAR is not known. Physiologically important cellular trafficking of transcription factors mediated by protein-protein interactions has been demonstrated in other systems (12). NFκB is retained in the cyto-
plasm as a complex with IκB, which is degraded after activation of its phosphorylation by stimuli (25). The protein Mdm2 has been proposed to bind to and target p53 for nuclear export (26). Nuclear factor of activated T cells (NFAT) is retained in the nucleus by binding to calcineurin, which blocks the nuclear export signals present in NFAT (27). Likewise, HDAC4 is primarily cytoplasmic in a complex with a 14-3-3 protein in HeLa cells (28) and exogenously expressed silencing mediator of retinoic acid and thyroid hormone (SMRT) results in the rapid nuclear localization of HDAC4 (29). In addition, phosphorylation or dephosphorylation has been shown to be involved in PB induction of CYP2B gene expression by the inhibition of PB-induced nuclear localization by the PP2A phosphatase inhibitor okadaic acid (8) and inhibition of nuclear activation of CAR by calcium/calmodulin kinase inhibitors (30, 31). The relevant phosphorylated proteins in these processes have not been identified, but one possible target is a p160 coactivator. Activation of p160 coactivators by phosphorylation has been reported (32).

Such activation of p160 coactivators after PB treatment could result in p160-mediated activation of nuclear mCAR and contribute to the nuclear localization of mCAR after PB treatment just as overexpression of GRIP1 might activate mCAR and result in nuclear localization in untreated animals.

Mutation of the XRS motif had been shown to block PB-induced hCAR nuclear localization in hepatocytes in vivo (10). In the present paper such mutations inhibited the GRIP1-mediated nuclear accumulation of mCAR and also resulted in a cytoplasmic rather than the normal nuclear localization in HepG2 cells. These results indicate that mutation of the XRS disrupts a basic nuclear transport function. Because the XRS does not resemble any nuclear localization signals, it is likely that interaction of CAR with another protein that is required for nuclear translocation is disrupted by the mutations as has been proposed (10). Because the mutation is a loss of function, the interacting protein is not likely to be involved in cytoplasmic retention but rather in actively transporting CAR into the nucleus.

The present results also suggest that the XRS region is important for functions other than translocation. The XRS motif is within the ligand-binding domain, which is also involved in dimerization and transactivation (10, 21). Interaction of mCAR with GRIP1 was only slightly blocked by the L326A mutation, but dramatically, the interaction of mCAR with GRIP1 was only slightly blocked by the L326A mutation. In models of the ligand-binding domain of PXR, Leu-329 forms part of the surface of the ligand binding cavity, and Leu-322 and Leu-326 extend away from the cavity along helix 11, so effects of these mutants on ligand binding or activation are not unexpected (33). The L322A/L326A/L329A XRS mutant had no detectable activity in HepG2 cells, whereas the L326A XRS mutant retained substantial activity of 30–50% of wild type, even if an NLS sequence was added to target the mutant to the nucleus. Interestingly, neither mutant was active in hepatocytes in vivo, suggesting that the nuclear fluorescence observed with these mutants in vivo was associated with inactive forms of the GFP-CAR chimera. Previous studies reported that the fold increase in transactivation in HEK293 cells mediated by the p160 coactivator SRC1 was the same in wild type hCAR.
and single substitution XRS mutants equivalent to Leu-322, Leu-326, and Leu-329 in mCAR (10). The substantial activity of the L326A mutant is consistent with these studies. However, the lack of activity in the L322A/L326A/L329A triple mCAR mutant provides functional evidence that the cellular localization motif of the XRS region overlaps with ligand binding and activation motifs of mCAR.

Deletion of the C-terminal 8 amino acids of mCAR, which eliminates the AP-2 domain, also resulted in decreased GRIP1-mediated nuclear accumulation of mCAR. Because the GRIP1 interacts with CAR through the AP-2 (20), this result is expected but indicates that interactions of GRIP1 with other domains of mCAR are not sufficient for its nuclear localization.

In addition, the C-terminal deletion resulted in a pronounced shift from nuclear to cytoplasmic localization of mCAR in cultured HepG2 cells, and PB-induced nuclear accumulation of the mutant in vivo was significantly less than wild type. This result is inconsistent with studies with hCAR in which deletion of its AP-2 domain did not detectably change PB-induced nuclear localization (10). The reason for the difference is not clear but could be due to differences between human and mouse CAR. The decreased nuclear accumulation of the transcriptionally inactive mCAR AP-2 mutant, however, is consistent with the idea that activation of mCAR contributes to increased nuclear accumulation.

These results suggest that cellular trafficking of CAR is more dynamic than simple sequestration in the cytoplasm and nuclear translocation after treatment with PB-like inducers. There is strong evidence that CAR is retained in the cytoplasm in a complex with heat shock protein 90 and CCRP (15, 16) and that PB treatment results in association of PP2A with this complex. Because okadaic acid, an inhibitor of PP2A, blocks translocation, dephosphorylation by PP2A probably results in a change in the complex to favor the translocation of CAR into the nucleus. Increased nuclear import is therefore an important contributor to the nuclear accumulation of CAR after PB treatment. The present results suggest that increased retention or stability of activated mCAR in the nucleus may also contribute to the nuclear accumulation probably as a result of interaction with p160 coactivators and other activating proteins in the nucleus. A reasonable conclusion is that mCAR is constantly shuttling between the cytoplasm and nucleus with the steady state strongly favoring cytoplasmic localization in untreated animals and strongly favoring nuclear localization after PB treatment as a result of changes in the rates of both nuclear import and export of mCAR.

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