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Overexpression of the Rho-guanine nucleotide exchange factor ECT2 inhibits nuclear translocation of nuclear receptor CAR in the mouse liver

Fardin Hosseinpour, **Yoav Timsit**, **Chika Koike**[1], **Kenji Matsui**, **Yukio Yamamoto**, **Rick Moore**, and **Masahiko Negishi**[*]
Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, United States

Abstract

Various drugs such as phenobarbital (PB) trigger translocation of constitutive active/adrostane receptor (CAR) from the cytoplasm into the nucleus of mouse liver cells without directly binding to the receptor. We have now characterized the guanine nucleotide exchange factor epithelial cell-transforming gene 2 (ECT2) as a PB-inducible factor as well as a cellular signal that represses PB-triggered nuclear translocation of CAR. When CFP-tagged ECT2 was co-expressed with YFP-tagged CAR in the liver of *Car*$^{-/-}$ mice, ECT2 repressed CAR nuclear translocation. Coexpression of various deletion mutants delineated this repressive activity to the tandem Dbl homology/pleckstrin homology domains of ECT2 and to their cytosolic expression. CAR directly bound to the PH domain. Thus, ECT2 may comprise a part of the PB response signal regulating the intracellular trafficking of CAR.

Keywords

Nuclear receptor CAR; Phenobarbital; ECT2; Drug metabolism; Liver; Nuclear translocation

1. Introduction

As the major organ for drug detoxification and excretion, the liver is endowed with the capability of inducing drug metabolizing enzymes and transporters in response to drug exposures. Upon activation by drugs such as phenobarbital (PB) and phenytoin, constitutive active/adrostane receptor (CAR) translocates from the cytoplasm to the nucleus in which the receptor forms a heterodimer with retinoid X receptor (RXR) to up-regulate transcription of the genes that encode drug metabolizing enzymes such as cytochromes P450 and the drug transporters [1-5]. The function of CAR has now been extended to the regulation of drug-induced repression of hepatic gluconeogenesis, by cross talking with the insulin response FoxO1 transcription factor to repress the genes such as *glucose-6-phosphatase* and *phosphoenoylpyruvate carboxykinase 1* [6,7]. In liver during regeneration, CAR up-regulates the expression of the *deiodinase 1* gene increasing thyroid hormone activity [8]. Also CAR is characterized as the essential factor for PB promotion of development of hepatocellular carcinoma [9]. Thus, deciphering the molecular mechanism of CAR activation by drugs is now critical for us to understand the receptor-mediated drug effects on liver functions and diseases.

[*]Corresponding author. Fax: +1 919 541 0696. E-mail address: negishi@niehs.nih.gov (M. Negishi).
[1]JSPS Research Fellow in the Biomedical and Behavioral Research Program at NIH.

Drug activation of CAR begins with the nuclear translocation from the cytoplasm of liver cells into the nucleus [10]. Unlike the nuclear steroid hormone receptors, for which their nuclear translocation is dictated by direct ligand binding, CAR is unique in which its nuclear translocation occurs without direct binding of its activators [11,12]. Although a cellular signal pathway may be present to retain CAR in the cytoplasm, no such a signal has yet been identified. epithelial cell-transforming gene 2 (ECT2) was originally cloned from epithelial cells and was characterized as a guanine nucleotide exchange factor bearing oncogenic activity [12-19]. We have linked ECT2 with CAR based on observations obtained in two independent analyses: (1) cDNA microarray analysis of wildtype and *Car*$^{-/-}$ mice after hepatectomy and PB treatment; and (2) looking for genes up-regulated during PB-promoted development of liver tumors [9]. Given these serendipitous findings, we pursued more detailed studies of ECT2, examining whether or not it regulates drug-induced nuclear translocation of CAR. By tail vein injection of expression plasmid DNAs, fluorescent protein tagged-ECT2 and its deletion mutants were directly co-expressed with CAR in mouse livers of *Car*$^{-/-}$ mice. ECT2 and CAR were also co-expressed in HepG2 cells for co-immunoprecipitation assays to define the molecular basis for their interaction. Thus, here we present experimental evidence that ECT2 directly interacts with CAR in the cytoplasm of liver cells to repress PB-induced CAR nuclear translocation.

2. Materials and methods

2.1. Materials

1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), anti-Flag HRP and anti-Flag M2-agarose were obtained from Sigma (St. Louis, MO); goat polyclonal GFP (HRP) antibody from Abcam Inc. (Cambridge, MA); Rabbit anti-hECT2 from Santa Cruz Biotechnology; and Complete Mini protease inhibitor cocktail tablets from Roche Diagnostics GmbH (Indianapolis, IN).

2.2. Plasmids

pEYFP-c1-mCAR and pEYFP-c1-mPXR were previously constructed [20,21]. Full-length cDNA of ECT2 was PCR-amplified from RNAs prepared from HepG2 cells using appropriate 5′-primer having an in-frame EcoRI site at the 5′-end and 3′primer bearing the KpnI site at the 3′-end. The amplified cDNA was digested by these two enzymes and cloned into pECFP-c1 (BD Biosciences) to produce pECFP-c1-ECT2. Using pECFP-c1-ECT2 as a template and a Quik-Change Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX), the expression plasmids for various ECT2 fragments were constructed: pECFP-c1-NS (amino acid residues 1-150), pECFP-c1-Dbl homology (DH)/pleckstrin homology (PH) (150-250), pECFP-c1-DH/PH/C (150-300), pECFP-c1-DH (150-200) and pECFP-c1-PH (200-250). To add a nuclear localization signal (NLS) sequence to the DHPH fragment, its cDNA was PCR-amplified using the appropriate 5′ primer and the 3′ primer containing the BamHI site at the 3′-end and cloned into pECFP-Nuc expression vectors carrying simian virus 40 large T antigen (BD Biosciences), to construct pECFP-Nuc-DH/PH. The BamHI and EcoRI fragments of NS, DH, PHC and mCAR were inserted at the BamHI and EcoRI sites of pACT or pBIND vector (Promega Co., Madison, WI). All plasmids were verified by nucleotide sequencing.

2.3. Immunoprecipitation

Anti-Flag M2-agarose (40 μl) was added to cytosol (1-2 mg of protein) prepared from HepG2 cells and incubated at 4 °C for 16 h. The remaining procedure was carried out as described previously [21].

2.4. Western blot

Western blot was carried out as described previously [21]. Membranes were incubated for 1 h at 25 °C with HRP-conjugated goat anti-GFP polyclonal antibody or overnight with rabbit anti-hECT2 primary antibody followed by 1 h incubation with HRP-conjugated donkey anti-rabbit IgG secondary antibody.

2.5. Animal experiments

Adult male *Car*$^{+/+}$ and *Car*$^{-/-}$ mice were used for all experiments. Cytosolic fractions and nuclear extracts were prepared mouse livers according to published methods [22,23]. Direct expression of ECT2 and/or CAR and analysis of their expression plasmids were performed as described previously [20].

2.6. Mammalian two-hybrid assays

HepG2 cells were seeded into 24-well plates (1×10^5 cells/well) and were co-transfected with pG5 (0.1 μg/well), pBIND-mCAR (0.2 μg/well) and pACT-ECT2 (0.2 μg/well). Total amount of plasmids transfected was adjusted by adding empty vector. Twenty-four hours after transfection, the cells were washed with PBS, supplemented with fresh serum-free medium and treated with 250 nM TCPOBOP or vehicle for 24 h prior to luciferase activity analysis. Luciferase activities were measured in cell lysates using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) as described previously [23].

2.7. Real-time PCR

Liver was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) to prepare total RNA according to the manufacturer's instructions. Two micrograms of RNA was used as a template for cDNA synthesis using the SuperScript first strand synthesis system (Invitrogen, Carlsbad, CA). One-twentieth of the cDNA was used for realtime PCR with an ABI Prism 7700 sequence detector using *Taq*Man Universal PCR reaction mix, and primers for mouse *ECT*2 (Mm00432964, Applied Biosystems, Foster City, CA) or *CYP2B10* [26] genes were used for amplification. Amplification of the *glyceraldehyde-3-phosphate* gene was used as a normalization control.

3. Results and discussion

3.1. CAR-mediated induction of ECT2

We first examined induction of ECT2 by PB in the livers of normal mice. Treatment with PB for 24 h resulted in a 3-fold induction of ECT2 mRNA in the wildtype but not in the *Car*$^{-/-}$ livers (Fig. 1A). These results indicate that ECT2 is a PB-inducible and CAR-regulated factor. *Car*$^{+/+}$ mice were treated by PB for 6 and 24 h, from which cytosolic fractions were prepared to examine the levels of ECT2 protein. Western blot analysis revealed that 6 h PB treatment increased ECT2 protein levels in both cytosolic and nuclear fractions (Fig. 1B). The cytosolic levels of ECT2 remained elevated after 24 h treatment, while no detectable ECT2 was observed in the nuclear factions.

3.2. Inhibition of CAR nuclear translocation by ECT2

ECT2 is known to regulate intracellular protein trafficking [19]. Therefore, we examined whether ECT2 can regulate nuclear translocation of CAR in mouse liver. For this, *Car*$^{-/-}$ mice were tail vein-injected with the appropriate plasmids to give liver expression of YFP-CAR with or without co-expression of CFP-ECT2. Confocal analysis of liver sections from these plasmid-injected mice revealed that YFP-CAR was localized in the cytoplasm, and upon PB treatment CAR translocated to the nucleus (Fig. 3A). However, when CFP-ECT2 was co-expressed with YFP-CAR, the CFP-CAR remained localized in the cytoplasm even after PB

treatment (Fig. 3B). Analysis of one hundred PB-treated hepatocytes revealed that the number of the hepatocytes localizing CAR in the nucleus decreased by 50% by co-expression of CFP-ECT2 (Fig. 3C). This inhibition of CAR nuclear translocation by ECT2 appeared to be specific to CAR, since CFP-ECT2 was found not to affect intracellular localization of pregnane X receptor (PXR) in the liver in the presence and absence of the PXR agonist pregnenolone-16α carbonitrile (PCN) (Fig. 3D,E). It remains an interesting question whether the specificity is originated from the differences in the molecules of the two receptors and/or in the signals generated by PB and PCN.

3.3. Delineation of the CAR binding site of ECT2

Given the possibility that ECT2 might bind to CAR in repressing the nuclear translocation, co-immunoprecipitation (co-IP) and mammalian two-hybrid analyses were performed to determine the region of ECT2 responsible for this interaction. Based on the subdomain structure of ECT2 (Fig. 2), expression plasmids encoding single or multiple subdomains were constructed. In co-I P experiments, pECFP-c1-NS, pECFP-c1-NS/DH, pECFP-c1-DH/PH, pECFP-c1-DH, or pECFP-c1-PH was co-transfected with Flag-mCAR in HepG2 cells and cytosols were prepared. Using anti-Flag M2-agarose Flag-CAR was purified, and associated ECT2 protein was detected by an anti-CFP antibody. The results showed that the PH domain alone is sufficient for ECT2 to interact with CAR (Fig. 4A). In mammalian two-hybrid assay analysis, pBIND-mCAR was co-transfected pACR-NS, pACR-DH or pACR-PH/C. The 2-fold increase in the luciferase activity with CAR and ECT2-PH/C co-transfection confirmed the interaction of ECT2's PH domain with CAR (Fig. 4B).

3.4. Requirement of DH domain in repressing CAR translocation

In order to investigate the role of ECT2 in repressing PB-dependent CAR nuclear translocation, a single CFP-tagged ECT2 deletion construct (NS, DH/PH, DH/PH/C, DH, or PH) was co-expressed with YFP-CAR in *Car*$^{-/-}$ mouse liver, followed by PB treatment. The percentage of cells with nuclear-localized YFP-CAR was then determined, normalized to the percentage of cells with nuclear-localized YFP-CAR in mice not co-expressing CFP-tagged ECT2. Of all the CFP-ECT2 deletion constructs, expression of the tandem DH/PH construct caused the greatest reduction (by approximately 90%) in PB-elicited YFP-CAR nuclear translocation (Fig. 5). In contrast to the strong inhibition by the DH/PH, expression of either the DH or PH domain alone did not inhibit the YFP-CAR nuclear translocation. These data suggest that the DH and PH domains must both be present for ECT2 to inhibit the nuclear translocation of CAR. Interestingly, the presence of the carboxyl (C) domain attenuated the inhibitory effect of the DH/PH on CAR translocation as revealed by only a 50% inhibition of translocation by full-length ECT2 and the ECT2-DH/PH/C.

3.5. Cytoplasmic localization of the DH/PH

When its expression plasmid was injected via mouse tail vein, CFP-DH/PH was exclusively expressed in the cytoplasm of mouse hepatocytes and this cytoplasmic localization was unaffected after the mouse being treated with PB (Fig. 6). To answer the question whether the cytoplasmic localization was required for DH/PH to inhibit the PB-induced nuclear localization of CAR, the expression plasmid pECFP-Nuc-DH/PH was constructed to force the DH/PH localizing in the nucleus by adding a nuclear translocation signal (NLS) (Fig. 6). CFP-Nuc-DH/PH by itself did not carry CAR into the nucleus, since co-expressed YFP-CAR was retained in the cytoplasm. Moreover, unlike co-expression of CFP-DH/PH that effectively inhibited PB-induced nuclear translocation of YFP-CAR, the co-expression CFP-Nuc-DH/PH did not inhibit this translocation. Cell counting revealed that 70% of PB-treated hepatocytes localizing YFP-CAR in the nucleus. These results equivocally show that DH/PH must be present in the cytoplasm to inhibit PB-induced CAR nuclear translocation.

3.6. No repression by ECT2 of CAR-mediated transcription

Unlike CAR in non-induced mouse liver, CAR spontaneously accumulates in the nucleus of HepG2 cells [10,11]. Therefore, we used HepG2 cells to examine whether ECT2 directly represses CAR-mediated transcriptional activation. For this, the $(NR1)_5$-tk-luciferase reporter and pEYFP-c1-mCAR plasmids were co-transfected with pECFP-c1-ECT2, pECFP-c1-DH/PH, or pECFP-c1-Nuc-DH/PH. The CAR agonist TCPOBOP activated the NR1 reporter activity, and neither fulllength ECT2 nor any of the deletion mutants repressed the CAR-mediated activation of $(NR1)_5$-tk-luciferase reporter (Fig. 7). These results indicate that ECT2 does not inhibit the activation function of CAR at the level of transcription in the nucleus.

4. Conclusion

Based on our findings, we propose that ECT2 may constitute a part of the cellular mechanism repressing PB activation of CAR by inhibiting the nuclear translocation of the receptor in the mouse hepatocytes. In addition to the cytoplasm and the nucleus, CAR also localizes to other compartments, most notably the cell membrane [25]. Furthermore, the nuclear level of CAR peaks in the liver 3-6 h after PB injection [10], and we have found that a second treatment PB injection at 24 h after the first treatment no longer accumulates CAR in the nucleus (data not shown). These observations imply the possibility that hepatocytes are endowed with a negative feedback mechanism repressing PB-induced nuclear translocation of CAR and regulating CAR-mediated transcription of hepatic genes. Growth factors such as hepatocyte growth factor repress the CAR nuclear translocation in mouse primary hepatocytes [26], which may, in part, be due to the fact that they induce ECT2 [18]. Thus, PB may trigger the negative feedback to repress CAR nuclear translocation though growth factor-ECT2 pathway.

Abbreviations

CAR, constitutive active/adrostane receptor; PXR, pregnane X receptor; RXR, retinoid X receptor; ECT2, epithelial cell-transforming gene 2; PH, pleckstrin homology; DH, Dbl homology; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; PB, phenobarbital.

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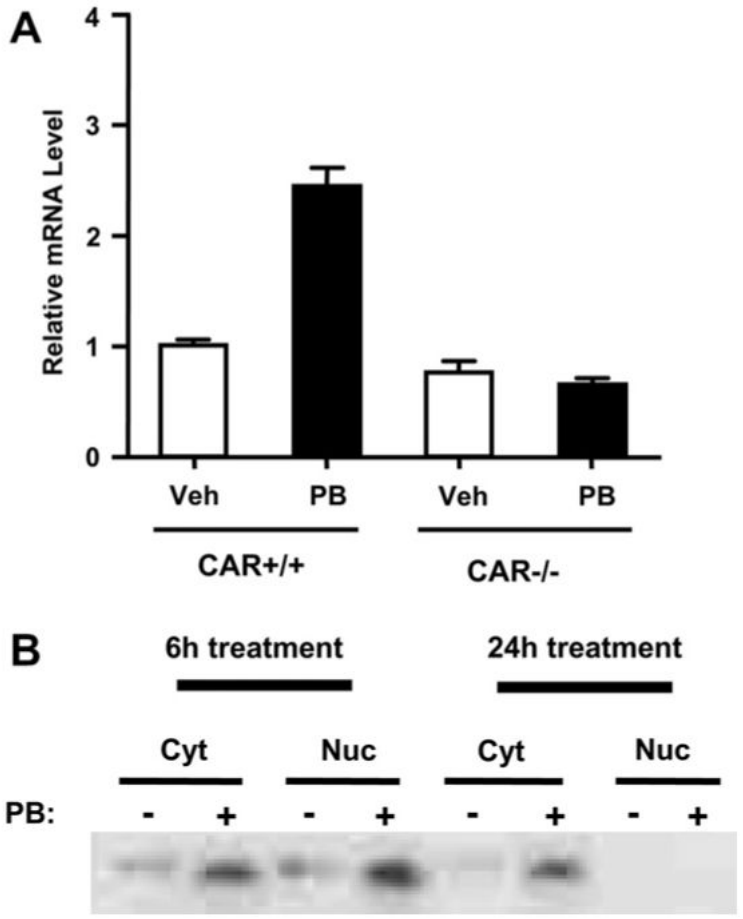

Fig. 1.

PB induction of ECT2 in the mouse liver. (A) PB (closed bar, 100 mg/kg body weight) or vehicle (open bar, saline) was intraperitoneally injected into *Car*$^{-/-}$ and *Car*$^{+/+}$ mice. After 24 h, total liver RNA was isolated and subjected to real-time PCR as described in Section 2. Each sample was performed in triplicate. (B) Liver cytosols were prepared from *Car*$^{+/+}$ mice at 6 and 24 h after intraperitoneal injection of PB (100 mg/kg) and were subjected to immunoblotting analysis using anti-ECT2 antibody.

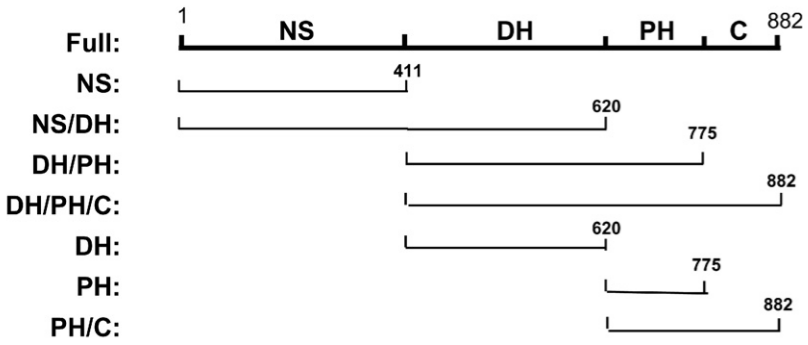

Fig. 2.
Schematic representations of domain structure of ECT2 and of the deletion mutants. This domain structure is depicted based on information published by Solski et al. [24].

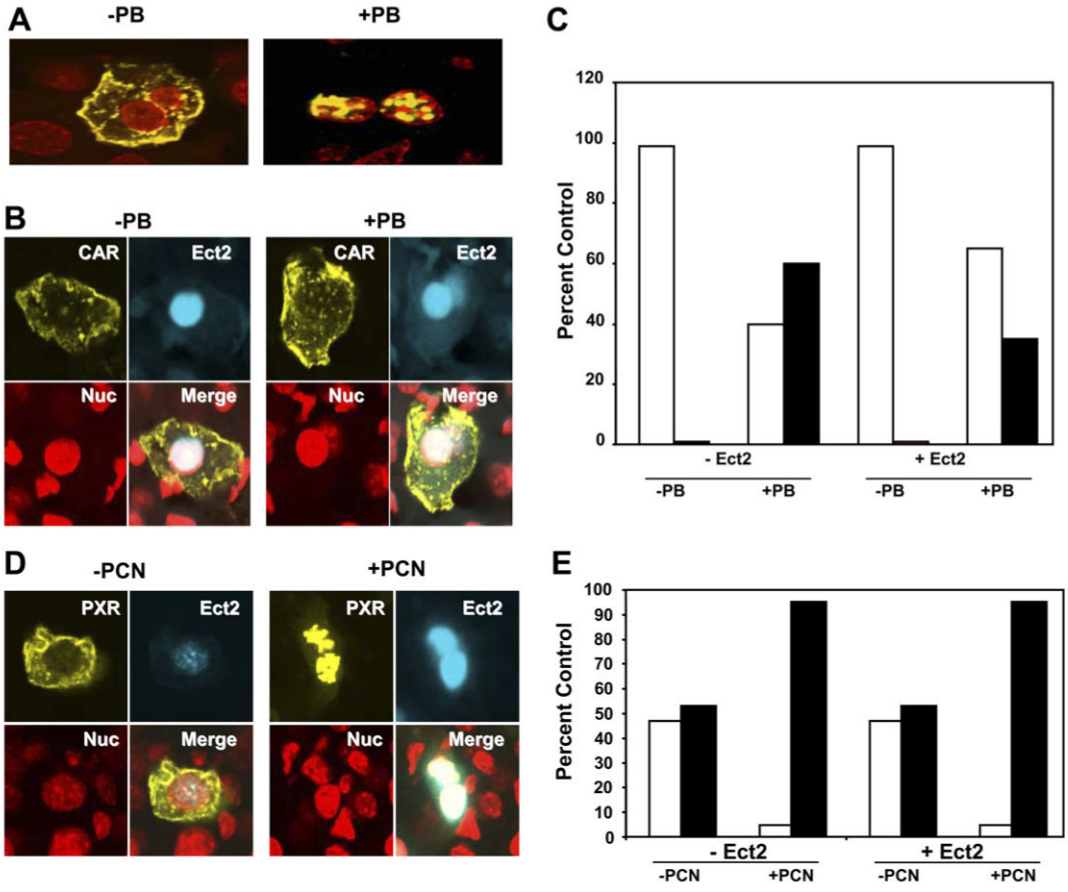

Fig. 3.

ECT2 inhibition of CAR nuclear translocation in the liver. (A) *Car*$^{-/-}$ mice were trail vein-injected with the expression plasmid of YFP-CAR and were treated with saline (-PB) or PB (+PB) as described in Section 2. Liver sections were prepared and examined by confocal microscopy for CAR expression (yellow) and Hoechest S33258 was used to stain nuclei (red). (B) *Car*$^{-/-}$ mice were trail vein-injected with the expression plasmids of YFP-CAR and CFP-ECT2 and were treated with saline (-PB) or PB (+PB). Liver sections were prepared and examined by confocal microscopy for CAR expression (yellow) and ECT2 expression (blue). Hoechest S33258 was used to stain nuclei (red). (C) One hundred hepatocytes expressing both CAR and ECT2 were counted, and CAR subcellular localization was categorized into two groups: cytoplasmic (open bars) and nuclear (closed bars). (D) and (E) YFP-PXR localization and effects of PCN treatment were assessed, using the same analysis as in (B) and (C).

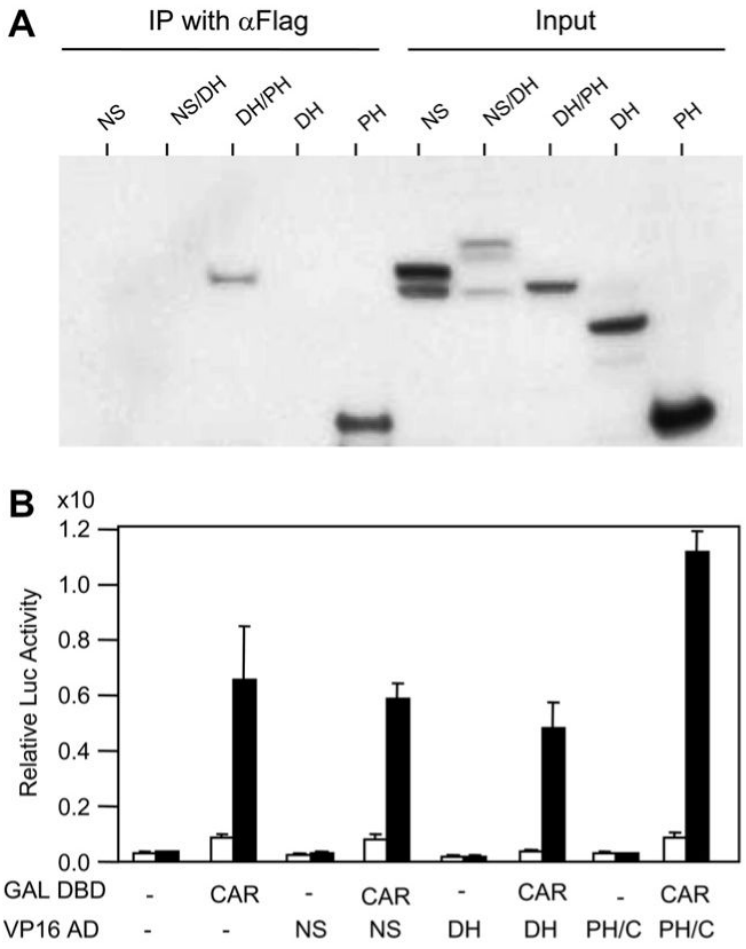

Fig. 4.

Delineation of the CAR binding site to PH domain. (A) Various CFP-tagged ECT2 fragments were co-expressed with Flagtagged mCAR in HepG2 cells as described in Section 2. Using HepG2 cell cytosol (1 mg protein), co-immunoprecipitation was carried out by anti-Flag antibody-conjugated agarose and was subjected to immunoblot analysis by anti-Flag HRP antibody. Input samples were 5% of the cytosol protein used for co-immunoprecipitations. (B) Mammalian two hybrid assays to show the interaction of CAR with PH domain. All experiments were performed as described in Section 2. The luciferase activity levels are indicated as the means ± S.D. of triplicate determinations, and shown is a representative result obtained in three independent experiments.

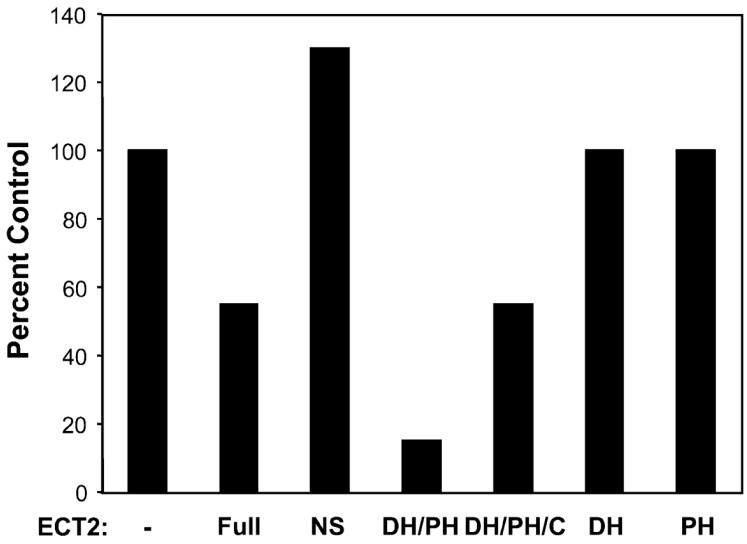

Fig. 5.

Delineation of the inhibitory activity to the DH/PH. YFP-tagged CAR and a CFP-tagged ECT2 were co-expressed in the *Car*$^{-/-}$ livers by tail-vein injecting their expression plasmids. Livers were removed from these mice after PB treatment and sections prepared and were examined by confocal microscopy. The number of hepatocytes having predominately nuclear YFP-CAR localization (out of 100 total hepatocytes) was divided by the number of cells expressing CAR in the nucleus (out of 100 total hepatocytes), giving a percentage. The percentage of cells with nuclear CAR when ECT2 was not co-expressed was set as 100%.

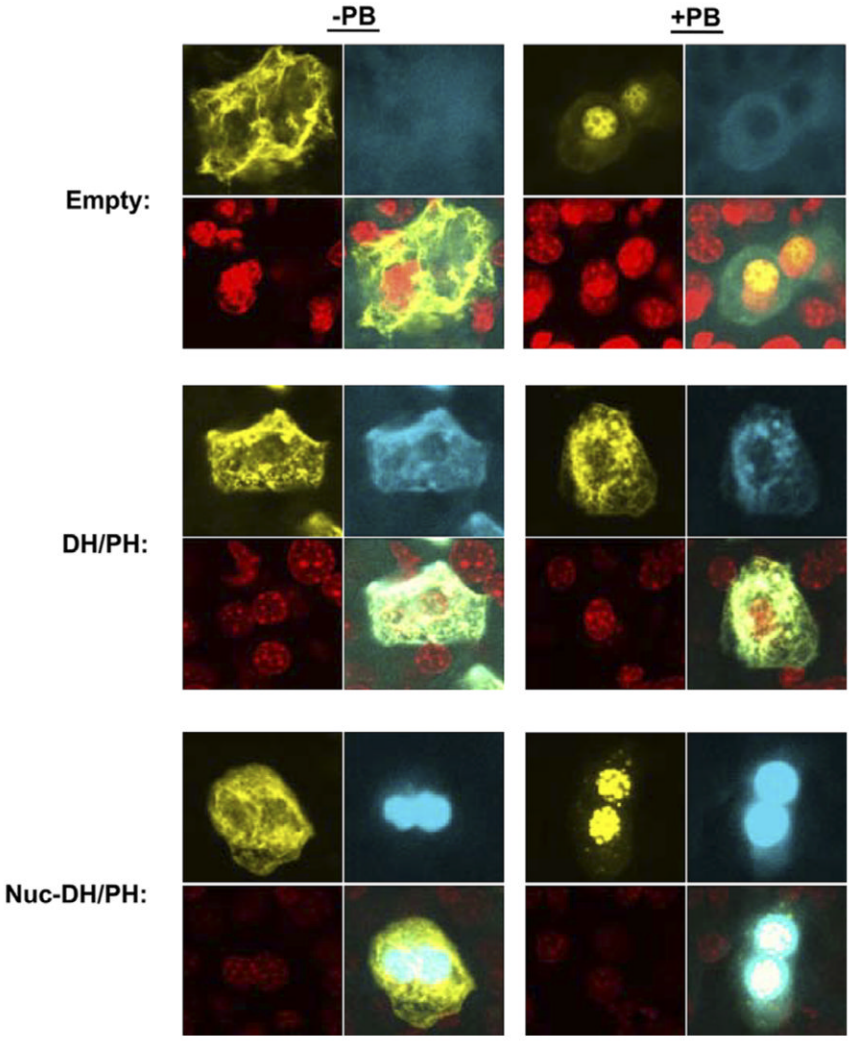

Fig. 6.

Cytoplasmic localization-dependent inhibition of CAR translocation by ECT2. YFP-tagged mCAR and either CFP-tagged DH/PH or CFP-tagged Nuc-DH/PH were co-expressed in the liver of *Car*$^{-/-}$ mice by tail-vein injection of their expression plasmids as described in Section 2. Mice were treated with saline (-PB) or PB (+PB), and liver sections were then prepared and examined by confocal microscopy for mCAR expression (yellow) and ECT2 expression (blue). Hoechest S33258 was used to stain nucleus (red).

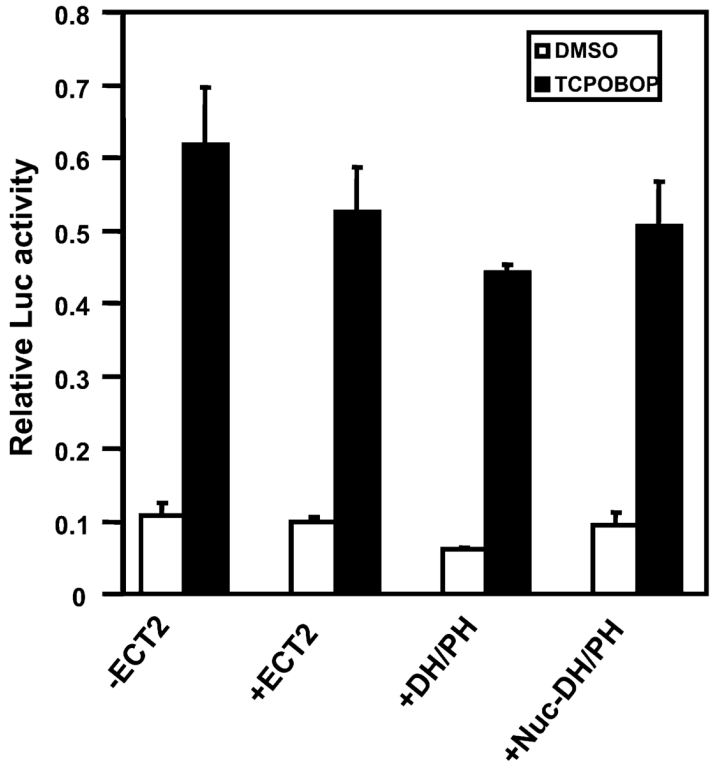

Fig. 7.

No repression by ECT2 of CAR-mediated transactivation. HepG2 cells were transfected with pECFP-c1-ECT, pECFP-c1-DH/PH, pECFP-c1-Nuc-DH/PH or empty pECFP-c1 with both pEYFP-c1-CAR and (NR1)5-tk-luciferase reporter plasmids. Twenty-four hours after transfection, the cells were treated with 250 nM TCPOBOP or DMSO for an additional 24 h and luciferase activities were determined as described in Section 2. Firefly luciferase activity was normalized against *Renilla reniformis* luciferase activity, and the means ± standard deviation was calculated based on triplicate determinations. Shown is a representative result in three independent experiments.