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

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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.

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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 phosphoenolpyruvate carboxykinase I [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.

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.

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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]
2. Materials and methods

2.1. Materials
1.4-Bis[2-(3,5-dichlorophenyl)oxy]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-i-ECT2 from Santa Cruz Biotechnology; and Complete Mini protease inhibitor cocktail tablets from Roche Diagnostics GmbH (Indianapolis, IN).

2.2. Plasmids
pEYFP-c1-NSCAR 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 pEYFP-c1 (BD Biosciences) to produce pEYFP-c1-ECT2. Using pEYFP-c1-ECT2 as a template and a Quick-Change Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX), the expression plasmids for various ECT2 fragments were constructed: pEYFP-c1-NS (amino acid residues 1–150), pEYFP-c1-Dbl homology (DH)/pleckstrin homology (PH) (150–250), pEYFP-c1-DH/PH/C (150–300), pEYFP-c1-DH (150–200) and pEYFP-c1-PH (200–250). To add a nuclear localization signal (NLS) sequence to the DH/PH fragment, its cDNA was PCR-amplified using appropriate 5’ and 3’ primers and cloned into pEFP-c1 (BD Biosciences) to produce pEFP-c1-ECT2. Using pEFP-c1-ECT2 as a template and a QuikChange Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX), the expression plasmids for various ECT2 fragments were constructed: pEFP-c1-NSCAR and pEFP-c1-mPXR were previously constructed [20,21].

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-i-ECT2 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 ptg5 (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 real-time PCR with an ABI Prism 7700 sequence detector using TaqMan Universal PCR reaction mix, and primers for mouse ECT2 (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 fractions.

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.

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 tail 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 Hoechst S33258 was used to stain nuclei (red). (B) Car−/− mice were tail 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). Hoechst 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).
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-IP 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<sup>−/−</sup> 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-elicted 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 TCPO-BOP activated the NR1 reporter activity, and neither full-length 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.

References


