CAR2 displays unique ligand binding and RXRα heterodimerization characteristics

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ABSTRACT

The constitutive androstane receptor (CAR, NR1I3) regulates the expression of genes involved in all phases of xenobiotic metabolism. Alternative splicing of the human CAR transcript yields an array of unique mRNAs. One form, termed CAR2, contains an additional 4 amino acids (SPTV) that are predicted to reshape the ligand-binding pocket. Results from the current studies demonstrate a marked, ligand-independent, CAR2-mediated transactivation of reporters derived from the natural CYP2B6 and CYP3A4 gene promoters. Over expression of RXRα is critical for achieving these effects. CAR2 interaction with SRC-1, assessed by mammalian 2-hybrid experiments, is similarly dependent on RXRα. Mutagenesis of S233 (SPTV) to an alanine residue yielded a receptor possessing higher constitutive activity. Alternatively, mutating S233 to an aspartate residue drastically reduced CAR2's transactivation capacity, a result that correlates to their ability to interact with RXRα, and to recruit SRC-1 in an inverse-agonist regulated manner. Together, these results demonstrate a robust RXRα-dependent recruitment of coactivators and transactivation by CAR2. In addition, CAR2's novel dose response to clotrimazole and androstanol relative to the reference form of the receptor suggests that CAR2 may be regulated by a unique set of ligands.

INTRODUCTION

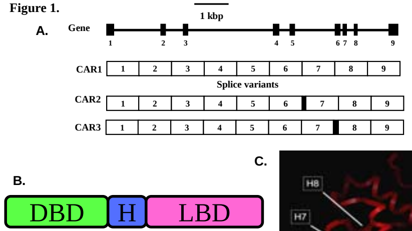
The constitutive androstane receptor (CAR, NR1I3) is a nuclear hormone receptor predominantly expressed in liver (1). CAR is implicated as a regulator of the metabolism of xenobiotics, drugs , carcinogens , steroids , heme , bile acids and thyroid hormone. Further, CAR activity may impact cholesterol homeostasis and signaling pathways that control food consumption. In the absence of an activating agent, e.g., the prototypical inducer - phenobarbital, CAR is localized within the cytosol of hepatocytes. However, upon exposure to certain agents, including selective ligands, CAR translocates to the nucleus, heterodimerizes with RXRα and interacts with nuclear coregulators, such as SRC-1, thereby modulating target gene expression independent of bound ligand. Further ligand modulation of CAR's constitutive activity may occur by disrupting the receptor's ability to interact with coactivators. The battery of CAR target genes include members of all 3 phases of xeno/endobiotic metabolism and clearance, such as certain cytochrome P450s, UDP-glucuronosyltransferase, sulfotransferase, glutathione S-transferase, aldehyde dehydrogenase, and ABC transporter families. CAR response elements have been mapped to a number of human genes, including CYP2B6, CYP3A4 and CYP2C9.

Recently, we and others have described a number of mRNA splice variants of the human CAR gene that potentially represent a large expansion of the CAR proteome. One of the variant forms results from the use of an alternative splice acceptor site in intron 6, leading to the insertion of 12 additional nucleotides. This transcript encodes a protein containing an additional 4 amino acids (SPTV) that are predicted to extend helix 6 of the ligand binding domain and potentially affect the structure of the ligand binding pocket. We term this form of the receptor, CAR2 (CAR1 is the reference form of the receptor). CAR2 retains limited ability to transactivate CAR responsive reporters, a result that correlates with a reduced affinity for RXRα and a compromised ability to interact with DNA. Ligand studies of CAR2 demonstrated that clotrimazole deactivated the receptor, whereas CITCO produced a weak, albeit significant activation of CAR2 - a result that is contrary to mammalian-2 hybrid studies published separately. Notably, in transfected mouse hepatocytes, nuclear translocation of CAR2 is not observed following CITCO treatment .

Studies presented here demonstrate that CAR2 comprises ~25% of the total CAR transcripts and that CAR2 is stably expressed in a rabbit reticulocyte system as well as in transfected bacterial and mammalian cell lines. CAR2 constitutively transactivates promoters derived from the endogenous CYP2B6 and CYP3A4 genes. The CAR inverse agonists, androstanol and clotrimazole, differentially repress CAR2 relative to CAR1, at varying doses. CAR2 also appears to retain the ability to bind CITCO. Further, we report that RXRα cotransfection greatly enhances CAR2's ability to transactivate reporter constructs. Mammalian 2-hybrid studies revealed that in contrast to CAR1, CAR2's ability to interact with coactivators is largely dependent on RxRα. Finally, mutagenesis studies conducted on S233 of CAR2 reveal that this site plays an important role in the receptor's ability to transactivate reporters, heterodimerize with RxRα and recruit SRC-1.

RESULTS

Figure 1.



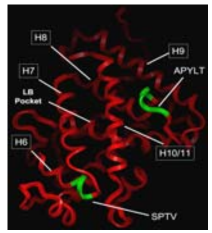
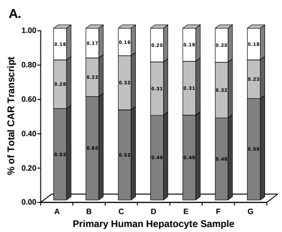


Fig. 1. Location and structure of the hCAR variants CAR2 and CAR3. (A) Location of nucleotide insertions for CAR2 and CAR3 compared to CAR1. (B) CAR domain structure. The insertions for CAR2 and CAR3 both reside in the ligand binding domain. (C) Ribbon structure of the CAR ligand binding domain. The 4 amino acid insertion of CAR2 (SPTV) is predicted to extend α-helix 6 in the C-terminal direction. The 5 amino acid insertion of CAR3 (APYLT) is predicted to extend the loop between α-helices 8 and 9.

Figure 2.



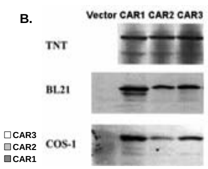
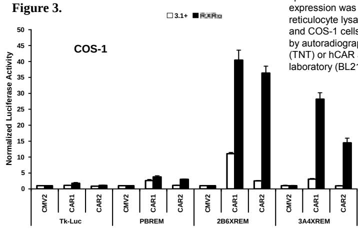


Fig. 2. Expression of CAR variants. (A) Quantitative RT-PCR was performed on RNA samples obtained from primary human hepatocytes. Absolute quantification of each isoform was determined with standard curves generated from plasmid DNA containing the specific isoform. (B) hCAR variant protein expression was observed in a rabbit reticulocyte lysate system as well as BL21 and COS-1 cells. Proteins were visualized by autoradiography of ^{35}S-labeled proteins (TNT) or hCAR antibody developed in our laboratory (BL21 and COS-1).

Figure 3.



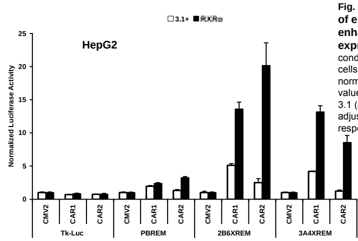


Fig. 3. CAR2 transactivation of endogenous promoters is enhanced by RXRα co-expression. Reporter assays conducted in COS-1 and HepG2 cells. Data are presented as normalized and adjusted luciferase values in which the activity of the 3.1 (or RXRα)/CMV2 group is adjusted to 1 for each respective response element.

RESULTS

Figure 4.

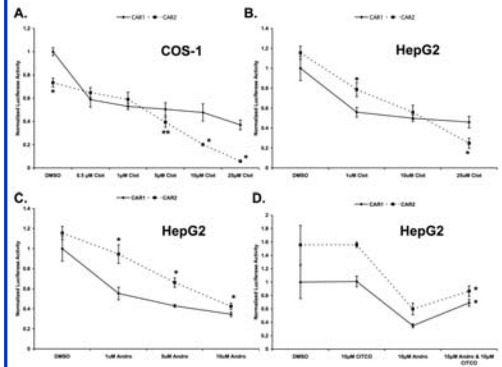


Fig 4. CAR2 displays altered activity in response to different concentrations of the inverse agonists clotrimazole and androstanol when compared to CAR1 and retains the capacity to bind CITCO. Reporter assays in COS-1 or HepG2 cells. All experiments used the 2B6XREM reporter construct and were co-transfected with RXRα. Data are presented as normalized luciferase values adjusted so that the CAR1/DMSO group in each panel is equal to 1. (A-C) An asterisk denotes data points where CAR2 is significantly different from CAR1 at a specific concentration of chemical. (mean +/- SD, n = 4, * = p < 0.01 ** = p < 0.05). (D) The asterisks denote that the activity of CAR1 and CAR2 is significantly greater in the groups treated with both androstanol and CITCO when compared to the respective androstanol only groups. (mean +/- SD, n = 4, * = p < 0.01)

Figure 5.

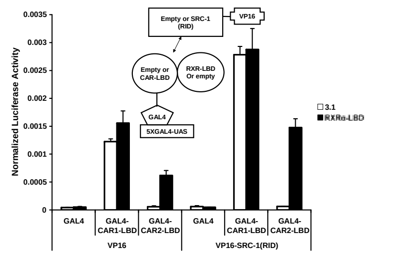


Fig 5. CAR2 recruitment of SRC-1 is RXRα-dependent. Mammalian 2-hybrid experiments performed in COS-1 cells. Data are presented as normalized luciferase values.

Figure 6.

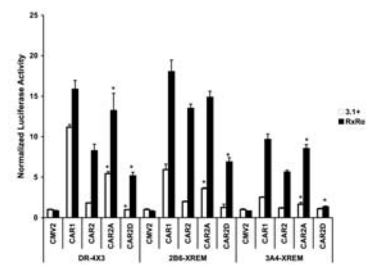
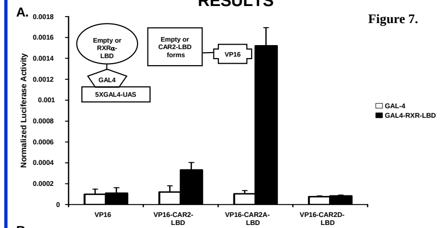


Fig 6. Mutation of the S233 site in CAR2 modifies its transactivation potential. Reporter assays in COS-1 cells. Data are presented as normalized and adjusted luciferase values in which the activity of the 3.1/CMV2 group is adjusted to 1 for each respective response element. Within group comparisons, data points are denoted with an asterisk to indicate that they deviate significantly from the relevant control (mean +/- SD, n = 4, p < 0.01).

RESULTS

Figure 7.



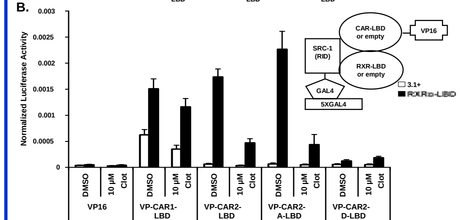


Fig. 7. Effects of S233 mutagenesis on heterodimerization and SRC-1 recruitment. Mammalian 2-hybrid experiments performed in COS-1 cells. Data are presented as normalized luciferase values. (A) Interaction between CAR2 and its S233A and S233D mutants with RxRα. (B) Interaction between CAR2 and its S233A and S233D mutants with SRC-1 in the presence and absence of RxRα and clot (clotrimazole).

CONCLUSIONS

- CAR2 is a naturally occurring hCAR splice variant that comprises ~25% of total CAR transcripts in hepatocytes and encodes a stable protein.
- Transactivation of reporter genes by CAR2 is ligand-independent and enhanced by co-expression of RXRα.
- CAR2 activity is more strongly repressed by the inverse agonist effects of clotrimazole, relative to CAR1, and retains the ability to bind CITCO.
- Recruitment of SRC-1 by CAR2 is dependent on the ligand binding domain of RXRα.
- Results of mutagenesis studies demonstrate that the S233 site of CAR2 is important for its ability to transactivate reporter genes, heterodimerize with RXRα and recruit SRC-1. These data suggest that a phosphorylation event at this location may regulate CAR2 activity.

REFERENCES

1. Baes M, Gulick T, Choi H S, Martinoli M G, Simha D and Moore D D (1994) A New Orphan Member of the Nuclear Hormone Receptor Superfamily That Interacts With a Subset of Retinoic Acid Response Elements. *Mol Cell Biol* **14**: pp 1544-1551.

ACKNOWLEDGEMENTS

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