

Agonist-dependent and Agonist-independent Transactivations of the Human Constitutive Androstane Receptor Are Modulated by Specific Amino Acid Pairs*

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The constitutive androstane receptor (CAR) is an interesting member of the nuclear receptor superfamily because of its exceptionally high constitutive activity due to ligand-independent interaction of the ligand-binding domain with co-activator proteins. This study compares the agonist-dependent and agonist-independent activities of human CAR with those of mouse CAR and the vitamin D receptor and demonstrates that the constitutive activity of CAR is mediated by at least three contacts between the amino acids of helix 12, partner amino acids in helices 4 and 11, and a charge clamp between helices 12 and 3. The stabilization of helix 12 by a contact between its C terminus and the lysine of helix 4 has the same impact in human and mouse CARs. In addition, the charge clamp between the glutamate in helix 12 and the lysine in helix 3 is also important for the constitutive activity of both receptor orthologs but less critical for the agonist-dependent stabilization of their respective helices 12. Interestingly, Cys-357 in mouse CAR has significantly more impact on the stabilization of helix 12 than does the orthologous position Cys-347 in human CAR. This deficit appears to be compensated by a more dominant role of Ile-330 in human CAR over Leu-340 in mouse CAR because it is more efficient than Cys-347 in controlling the flexibility of helix 12 in the presence of an agonist. The constitutive activity of other members of the nuclear receptor superfamily could be explained by a homologous hydrophobic interaction between large, non-polar amino acids of helices 11 and 12.

transducing the effects of small lipophilic compounds into a transcriptional response (1). The existence of a highly conserved DNA-binding domain and a structurally conserved ligand-binding domain (LBD) define the family (2). The LBDs of most NRs consist of 12 α -helices that form a characteristic three-layer sandwich (3). The last helix, helix 12, serves as a molecular switch by interacting in the agonistic conformation of the LBD with co-activator (CoA) proteins such as DRIP205/TRAP220, SRC-1/NCoA-1, TIF2/NCoA-2, and RAC3/NCoA-3 (4), which activate target gene transcription by remodeling chromatin or by providing a bridge between the NR and the basal transcription machinery (5). In the absence of ligand, NRs interact with co-repressor proteins such as NCoR, SMRT, and Alien (6). CoAs and co-repressors make direct contacts with a hydrophobic cleft on the surface of the NR LBD, and the ability to discriminate between both classes of co-regulators is determined by the position of helix 12 (7). This biphasic situation allows NR ligands to activate or repress the transcription of specific target genes.

Classical endocrine NRs are the receptors for estrogen, progesterone, testosterone, cortisol, aldosterone, $1\alpha,25$ -dihydroxyvitamin D_3 ($1\alpha,25$ (OH) $_2D_3$), thyroid hormone, and all-trans retinoic acid that show a very selective ligand binding with K_d -values in the order of 1 nM or lower (8). The “mouse trap” model (3) is based on the crystal structure of some of these NRs and proposes that helix 12 may act as a lid to the ligand-binding pocket of the LBD, which is closed when the NR interacts with CoAs and open when the receptor is in the repressed state. Adopted orphan NRs form another subclass within the NR superfamily that binds a variety of structurally diverse compounds with a relatively low affinity (K_d in the order of 1 μ M) (9). The crystal structures of two adopted NRs, the pregnane X receptor (PXR; NR1I2) (10) and the peroxisome proliferator-activated receptor (NR1C1-3) (11), indicated that helix 12 takes a continuum of positions that span the extremes of complete repression and complete activation, such that fairly minor changes in the position of the helix can have a large impact on the transcriptional activity of NRs. Another interesting adopted orphan NR is the constitutive androstane receptor (CAR; NR1I1) (12). This receptor is characterized by having an exceptionally high constitutive activity. CAR and PXR play key roles in the response to chemical stress and regulate an overlapping set of genes, some of which encode proteins such as cytochrome P450 monooxygenases (CYPs) that are involved in the detoxification of potentially harmful xenobiotics and endobiotics (13). Induction of these enzymes confers a higher metabolic capability to organisms and contributes to their defense mechanisms against xenochemical toxicity and carcinogenicity. The study of human CAR was complicated by a number of ligand-related issues. First, the CAR ligands 5-preg-

Nuclear receptors (NRs)¹ form a large family of transcription factors (48 human members) and have critical roles in nearly all aspects of vertebrate development and adult physiology by

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¹ The abbreviations used are: NR, nuclear receptor; $1\alpha,25$ (OH) $_2D_3$, $1\alpha,25$ -dihydroxyvitamin D_3 ; CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b](1,3)thiazole-5-carbaldehyde *O*-3,4-dichlorobenzyl)oxime; CoA, co-activator; CYP, cytochrome P450 mono-oxygenase; DR, direct repeat; GST, glutathione *S*-transferase; LBD, ligand-binding domain; PBREM, phenobarbital responsive enhancer module; PXR, pregnane X receptor; RE, response element; RXR, retinoid X receptor; RAC3, receptor-associated coactivator 3; SRC-1, steroid receptor coactivator 1; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene; TIF2, transcription intermediary factor 2; VDR, $1\alpha,25$ (OH) $_2D_3$ receptor.

nane-3,20-dione (agonist) and clotrimazole (inverse agonist) are also effective activators of the more widely expressed PXR. Second, some suspected CAR ligands such as phenobarbital were subsequently found to act in an indirect fashion. Finally, the study of CAR was further hindered by the fact that this receptor has unique ligand response profiles in different organisms. For instance, the hepatomitogen 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (14) is a potent murine CAR ligand but does not activate human CAR. Therefore, the recent identification of the imidazothiazole derivative 6-(4-chlorophenyl)imidazo[2,1-*b*](1,3)thiazole-5-carbaldehyde-*O*-3,4-dichlorobenzyl)oxime (CITCO) as a selective human CAR agonist (15) finally enabled a number of studies on the selective actions of the receptor.

NR-responsive genes are defined through the presence of particular binding sites, which are referred to as response elements (REs) in their promoter regions (16, 17). CAR has been shown to form heterodimers with the retinoid X receptor (RXR; NR2B1) on REs that are formed by a direct repeat (DR) of hexameric binding sites (18). CAR-RXR heterodimers bind optimally to DR4-type REs (19), which are also recognized by PXR, the vitamin D receptor (VDR; NR1I1), and a number of other members of the NR superfamily. An investigation of previously characterized CAR-responding genes indicated that a single CAR RE may be insufficient for mediating the regulatory role of the receptor and that, more likely, at least two CAR REs in close proximity to each other are necessary. These multiple CAR RE clusters are commonly called phenobarbital-responsive enhancer modules (PBREMs). The mouse *CYP2B10* (orthologue to human *CYP2B6*) gene contains two DR4-type REs with an additional binding site for the transcription factor NF-1 (20), whereas the PBREM of the human UDP-glucuronosyltransferase *IA1* gene is formed by three CAR REs, one of which binds CAR monomers exclusively (19).

This study aims to understand the critical impact of helix 12 of human CAR for both the constitutive and the agonist-dependent activity of the receptor. The relative impact of four different possibilities for the stabilization of helix 12 via amino acid contacts was assessed in comparison with human VDR and mouse CAR.

MATERIALS AND METHODS

Compounds—CITCO was obtained from Biomol (Copenhagen, Denmark), and TCPOBOP was synthesized and purified according to Honkakoski *et al.* (21). $1\alpha,25(\text{OH})_2\text{D}_3$ was kindly provided by L. Binderup (Leo Pharma, Ballerup, Denmark). $1\alpha,25(\text{OH})_2\text{D}_3$ was dissolved in 2-propanol, whereas the other compounds were dissolved in dimethyl sulfoxide (Me_2SO); further dilutions were made in Me_2SO (for *in vitro* experiments) or in ethanol (for cell culture experiments).

Protein Expression Vectors—Full-length cDNAs for human CAR (12), human VDR (22), and human RXR α (23) were subcloned into the T_7 /SV40 promoter-driven pSG5 expression vector (Stratagene). The full-length cDNA for mouse CAR (24) was subcloned into the T_7 /CMV promoter-driven pCMX expression vector. The point mutants of human and mouse CARs and human VDR were generated using the QuikChange point mutagenesis kit (Stratagene) and confirmed by sequencing. The helix 12 deletion mutants of human CAR and human VDR were created by introducing a stop codon at amino acid positions 342 and 413, respectively, in the proteins. The extensions of the helices 12 in human and mouse CARs by three amino acids were generated by a double mutant that converted the original stop codon into a coding triplet and the third downstream triplet into a stop codon. The same constructs were used for both the T_7 RNA polymerase-driven *in vitro* transcription/translation of the respective cDNAs and for the viral promoter-driven overexpression of the respective proteins in mammalian cells.

Glutathione S-transferase (GST) Fusion Protein Construct—The NR interaction domains of mouse SRC-1 (spanning amino acids 597–791) (25), human TIF2 (spanning amino acids 646–926) (26), and human RAC3 (spanning amino acids 673–1106) (27) were subcloned into the GST fusion vector pGEX (Amersham Biosciences).

Reporter Gene Constructs—One copy of the PBREM of the mouse *CYP2B10* gene promoter (containing two DR4-type REs) (20) and two copies of the idealized DR4-type RE (DR4(T/T)) or the first DR4 of the PBREM (NR1) were fused with the thymidine kinase (*tk*) minimal promoter driving the firefly luciferase reporter gene. For core sequences of the REs, see Fig. 1A.

In Vitro Translation and Bacterial Overexpression of Proteins—*In vitro* translated wild type or mutated human and mouse CARs, human VDR, and human RXR α proteins were generated by coupled *in vitro* transcription/translation using rabbit reticulocyte lysate as recommended by the supplier (Promega, Madison, WI). Protein batches were quantified by test translations in the presence of [^{35}S]methionine. The specific concentration of the receptor proteins was adjusted to ~4 ng/ μl after taking the individual number of methionine residues per protein into account. Bacterial overexpression of GST-SRC-1 $_{597-791}$, GST-TIF2 $_{646-926}$, GST-RAC3 $_{673-1106}$, or GST alone was obtained from the *Escherichia coli* BL21(DE3)pLysS strain (Stratagene) containing the respective expression plasmids. Overexpression was stimulated with 0.25 mM isopropyl- β -D-thio-galactopyranoside for 3 h at 37 °C, and the proteins were purified and immobilized on glutathione-Sepharose 4B beads (Amersham-Pharmacia) according to the manufacturer's protocol. Proteins were eluted in the presence of glutathione.

Gel Shift and Supershift Assays—Gel shift assays were performed with equal amounts (~10 ng) of the appropriate *in vitro* translated protein. The proteins were incubated for 15 min in a total volume of 20 μl of binding buffer (10 mM Hepes (pH 7.9), 150 mM KCl, 1 mM dithiothreitol, 0.2 $\mu\text{g}/\mu\text{l}$ poly(dI-C) and 5% glycerol). For supershift experiments, 0.3 μg of bacterially expressed GST fusion proteins (or GST alone as negative control) were added to the reaction mixture. Approximately 1 ng of ^{32}P -labeled, double-stranded oligonucleotides (50,000 cpm) corresponding to one copy of the DR4-type REs DR4(T/T) or NR1 were then added, and incubation was continued for 20 min at room temperature. Protein-DNA complexes were resolved by electrophoresis through 8% non-denaturing polyacrylamide gels in 0.5 \times TBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA (pH 8.3)) and quantified on a Fuji FLA3000 (Fuji, Tokyo, Japan) reader using Image Gauge software (Fuji).

Transfection and Luciferase Reporter Gene Assays—MCF-7 human breast cancer cells were seeded into 6-well plates (10^5 cells/ml) and grown overnight in phenol red-free Dulbecco's modified Eagle's medium supplemented with 5% charcoal-stripped fetal bovine serum. Plasmid DNA containing liposomes were formed by incubating 1 μg of a reporter plasmid and 1 μg of an expression vector for wild type or mutated human or mouse CAR or human VDR with 10 μg of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (Roth, Karlsruhe, Germany) for 15 min at room temperature in a total volume of 100 μl . After dilution with 900 μl of phenol red-free Dulbecco's modified Eagle's medium, the liposomes were added to the cells. Phenol red-free Dulbecco's modified Eagle's medium supplemented with 500 μl of 15% charcoal-stripped fetal bovine serum was added 4 h after transfection. At this time, NR ligands or control solvents were also added. The cells were lysed 16 h after the onset of stimulation using the reporter gene lysis buffer (Roche Diagnostics), and the constant light signal luciferase reporter gene assay was performed as recommended by the supplier (Canberra-Packard, Groningen, The Netherlands). The luciferase activities were normalized with respect to protein concentration, and induction factors were calculated as the ratio of the luciferase activity of ligand-stimulated cells to that of solvent controls.

RESULTS AND DISCUSSION

For a direct comparison of the transactivation level of the adopted orphan NR human CAR and the endocrine NR human VDR, the idealized DR4-type RE DR4(T/T), which is a potent binding site for both CAR and VDR-RXR heterodimers (19, 28), the PBREM of the mouse *CYP2B10* gene (29), and the downstream DR4-type RE of the PBREM (NR1) (30), was chosen (Fig. 1A). The PBREM, two copies of DR4(T/T), or twice the RE NR1 were fused with the *tk* promoter driving the luciferase gene. Reporter gene assays were performed with these constructs in the transiently transfected model cell line MCF-7 (Fig. 1B). On all three REs, CAR signaling showed a very comparable profile, which is characterized by the following: (i) no significant response to the agonist CITCO at endogenous NR levels (MCF-7 cells do not express CAR endogenously); (ii) a 4–8-fold increased basal expression due to CAR overexpres-

1.7-fold (Fig. 1C, middle and right panels). Residual CAR monomer binding was observed on the DR4(T/T) but not to the NR1 RE. This was due to the lack of an optimal CAR monomer binding motif in the NR1 RE (19). Finally, supershift assays were performed with the p160 CoA protein family members SRC-1, TIF2, and RAC3 interacting with the CAR-RXR and VDR-RXR heterodimers formed on NR1 (Fig. 1D). Because of the high amount of bacterially expressed CoA proteins (1.5 μ g), CAR-RXR heterodimers interacted with all three CoAs already in the absence of agonist. However, in the presence of CITCO the supershifts were found to be more intense. In contrast, VDR-RXR heterodimers complexed with SRC-1, TIF2, and RAC3 only in the presence of $1\alpha,25(\text{OH})_2\text{D}_3$, as was expected for a classical endocrine NR. Interestingly, the three CoAs were indistinguishable from each other in their interaction profiles with CAR or VDR. This finding has not yet been shown for CAR signaling, but it confirms our earlier observations on VDR signaling (8). Taken together, the results indicate that the natural DR4-type RE NR1 is well suited for comparing CAR and VDR transactivation both in living cells as well as *in vitro*. This finding is supported by a recent report on the binding to and activation by VDR-RXR heterodimers on the NR1 of the human CYP2B6 PBREM (31). Therefore, the RE NR1 and the CoA TIF2 were used as representatives throughout this study. In addition, the data showed that $1\alpha,25(\text{OH})_2\text{D}_3$ and the VDR have functional impact even on a classical CAR RE, which demonstrated the functional interference of the signaling of endocrine and adopted orphan NRs.

In contrast to most other adopted orphan and classical endocrine NRs, no structural data based on x-ray crystallography for CAR is available (32). However, CAR shares approximately the equal levels of amino acid sequence homology to its evolutionary closest neighbors PXR and VDR (33), such that a model for CAR could be built on the basis of the crystal structure data derived from either of these two NRs. A recently published computer-generated structural model of the LBD of mouse CAR (34) was based on the crystal structure of human PXR (10). Therefore, we also employed the coordinates of the PXR structure for visualizing critical amino acids in the LBD of human CAR (Figs. 2, A and B). In VDR, an effective contact with CoAs is achieved when the charge clamp formed by the positively charged Lys-246 (Lys-177 in human CAR; see Fig. 2A), and the negatively charged Glu-420 (Glu-345 in human CAR; see Figs. 2A and 4A) has an optimal distance of 19 Å (35). This finding is supported by fixing the position of helix 12 by an additional interaction between Phe-422 and His-397 (homologous to Cys-347 and Tyr-326 in human CAR; see Fig. 4A). In addition, His-397 is correctly positioned by a contact with the hydroxyl group of $1\alpha,25(\text{OH})_2\text{D}_3$ at carbon 25. In the absence of ligand, helix 12 is not fixed and does not allow efficient CoA contact. First, we investigated whether the homologous amino acids in human CAR have a similar impact on the function of this NR. Whereas the deletion of the whole helix 12 completely abrogated the effect of CITCO on human CAR transactivation in MCF-7 cells, the mutants K177A and E345A kept full and weak ligand inducibility, respectively (Fig. 2C, left panel). However, the basal activity of all three CAR mutants was reduced by 65–75% relative to the level observed with wild type human CAR (Fig. 2C, right panel). In supershift assays, wild type CAR showed reasonable interaction with the CoA TIF2 even in the absence of ligand (Fig. 2D). This complex formation was further increased by the addition of CITCO. It has to be noted that we used limiting amounts of bacterially expressed TIF2 (0.3 μ g), which affects the relative migration difference between the CAR-RXR-DNA and CAR-RXR-TIF2-DNA complexes but is necessary in order to observe significant ligand-specific effects

on NR-CoA interactions (compare also Fig. 1D). The constitutive interaction of CAR with TIF2 was blunted by each of the three mutants K177A, E345A, and Δ H12 (deletion of helix 12). Moreover, the ligand-induced complex formation between DNA-bound CAR-RXR heterodimers and CoA was completely inhibited by the deletion of helix 12. However, in the presence of CITCO, the CAR mutants K177A and E345A were still able to supershift with TIF2 (Fig. 2D). This ability indicates that both charge clamp amino acids contribute to the constitutive activity of human CAR by stabilizing the binding of CoA to the receptor in an agonist-independent fashion and are of minor importance for the ligand-dependent transactivation of human CAR. This finding agrees with our recent report on mouse CAR (36). For contrast, the homologous mutations in human VDR have the opposite effects. The individual mutation of the charge clamp amino acids Lys-246 and Glu-420, as well as the deletion of helix 12, completely blunted $1\alpha,25(\text{OH})_2\text{D}_3$ -induced transactivation in MCF-7 cells but had no statistically significant effect on the basal activity of VDR (Fig. 2E). In supershift assays, an interaction of DNA-bound VDR-RXR heterodimers could only be observed in the presence of ligand but not in its absence (Fig. 2F). This ligand-induced complex formation was abrogated completely by each of the three mutations.

In our previous findings about mouse CAR (36), the interaction of Cys-357 in helix 12 with Tyr-336 in helix 11 (Fig. 4A) had a critical impact on the stabilization of helix 12. We next sought to investigate the role of these amino acids in human CAR. Therefore, these amino acids as well as the orthologous amino acids Phe-422 and His-397 in VDR (see Fig. 4A) were mutated individually and assessed in reporter gene and supershift assays (Fig. 2, C and D). The mutation of tyrosine to alanine at position 326 in human CAR blunted the inducibility of human CAR by CITCO in MCF-7 cells, reduced the basal activity by >50%, and prevented interaction with TIF2. In contrast, the mutation C347A increased ligand inducibility by a factor of 1.5, reduced the basal activity of CAR by only 30%, abrogated the interaction with CoA in the absence of ligand, and reduced it in the presence of ligand (Fig. 2, C and D). In mouse CAR the homologous mutations Y336A and C357A both displayed a more drastic reduction of the basal activity of the receptor and the loss of interaction with TIF2. In addition, Y336A showed the same ligand inducibility, because wild type mouse CAR and C357A increased it by a factor of 2 (data not shown; compare also Ref. 36). The orthologous mutations in human VDR, H397A and F422A, both abrogated ligand inducibility and the interaction with CoA but did not affect basal activity of the receptor (Fig. 2, E and F). Taken together, in human CAR the impact of the Tyr-326/Cys-347 interaction was found to be less crucial than the Tyr-336/Cys-357 and His-397/Phe-422 interactions in mouse CAR and human VDR, respectively. In particular, Cys-347 was shown to be less important for the constitutive and agonistic action of human CAR than Cys-357 is for that of mouse CAR (36). The latter finding indicates that there are species-specific differences in the mechanisms of stabilization and that different amino acid contacts contribute differentially to stabilize helix 12 in human and mouse CARs.

A recent report on mouse CAR (34) indicated that an interaction of the negatively charged C terminus of helix 12 with the positively charged Lys-205 (Lys-195 in human CAR) in helix 4 may contribute to the stabilization of helix 12 (see Fig. 2B). To test this possibility, we mutated the respective lysine of helix 4 and the extended helix 12 by three amino acids in both human and mouse CARs. Both mutants reduced the constitutive activity of human and mouse CARs in MCF-7 cells by 55 and 70%, respectively, and completely blunted the ligand-independent *in*

FIG. 2. Impact of helix 12 for the ligand-independent and ligand-dependent transactivation and CoA recruitment of human CAR and VDR. *A*, a model of human CAR was created on the basis of the human PXR structure (1NRL), and the positions of helix 12 and the amino acids Lys-177 (*K177*) and Glu-345 (*E345*) are shown in relation to the CoA peptide. Possible interacting amino acids are highlighted in color (*red*, negatively charged; *blue*, positively charged; *green*, non-charged). *B*, detailed view of the position of the critical amino acids that stabilize helix 12 by interaction with helices 11 and 4. *C–F*, reporter gene assays were performed with extracts from MCF-7 cells that were transiently transfected with a luciferase reporter construct containing two copies of the DR4-type RE NR1 and expression vectors for wild type and mutated human CAR (*C*) or human VDR (*E*). Cells were treated for 16 h with solvent, 1 μ M CITCO (*C*), or 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ (*F*). Data were normalized to the basal activity of the respective receptor mutant (*left panels*). In addition, the basal activity of each mutant was normalized to that of the wild type receptor (*right panels*). *Columns* represent the mean of at least three experiments, and *error bars* indicate standard deviations. Two-tail, paired Student's *t* test was performed, and *p* values were calculated in reference to the respective solvent control (*left panels*) or the basal activity of wild type (*wt*) receptor (*right panels*) (*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001). Super-shift experiments were performed with equal amounts of *in vitro* translated wild type and mutated human CAR (*D*) or human VDR (*F*), RXR protein, and ^{32}P -labeled NR1-RE. CAR-RXR and VDR-RXR heterodimers were pre-incubated with either solvent, 1 μ M CITCO, or 1 μ M $1\alpha,25(\text{OH})_2\text{D}_3$, respectively. Equal amounts of bacterially expressed GST as a control (–) or GST-TIF2_{646–926} (+) were then added. Protein-DNA complexes were resolved from free probe through 8% non-denaturing polyacrylamide gels. Representative gels are shown (*D*). It has to be noted that limiting amounts of bacterially expressed TIF2 (0.3 μ g) were used, which affects the relative migration difference between the CAR-RXR-DNA and CAR-RXR-TIF2-DNA complexes but is necessary in order to observe significant ligand-specific effects on receptor-CoA interactions. Δ H12, deletion of helix 12.

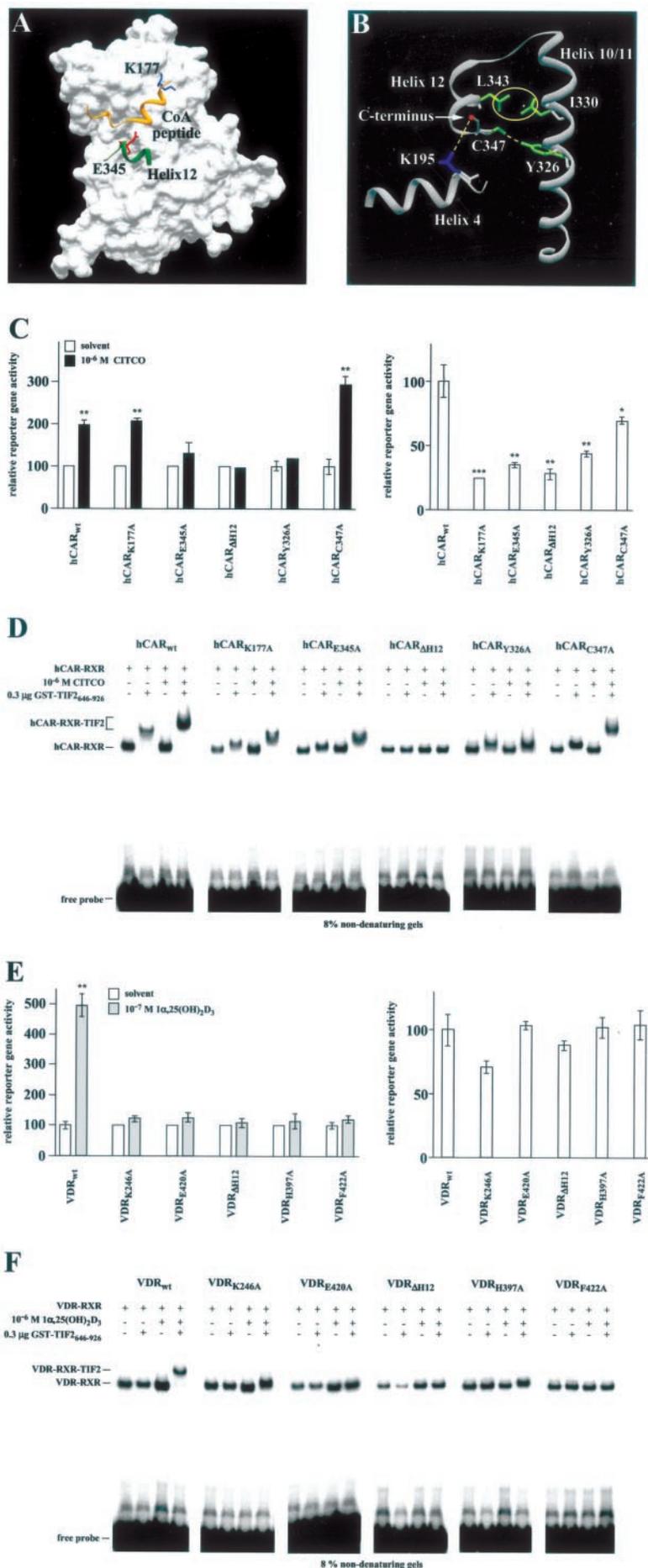
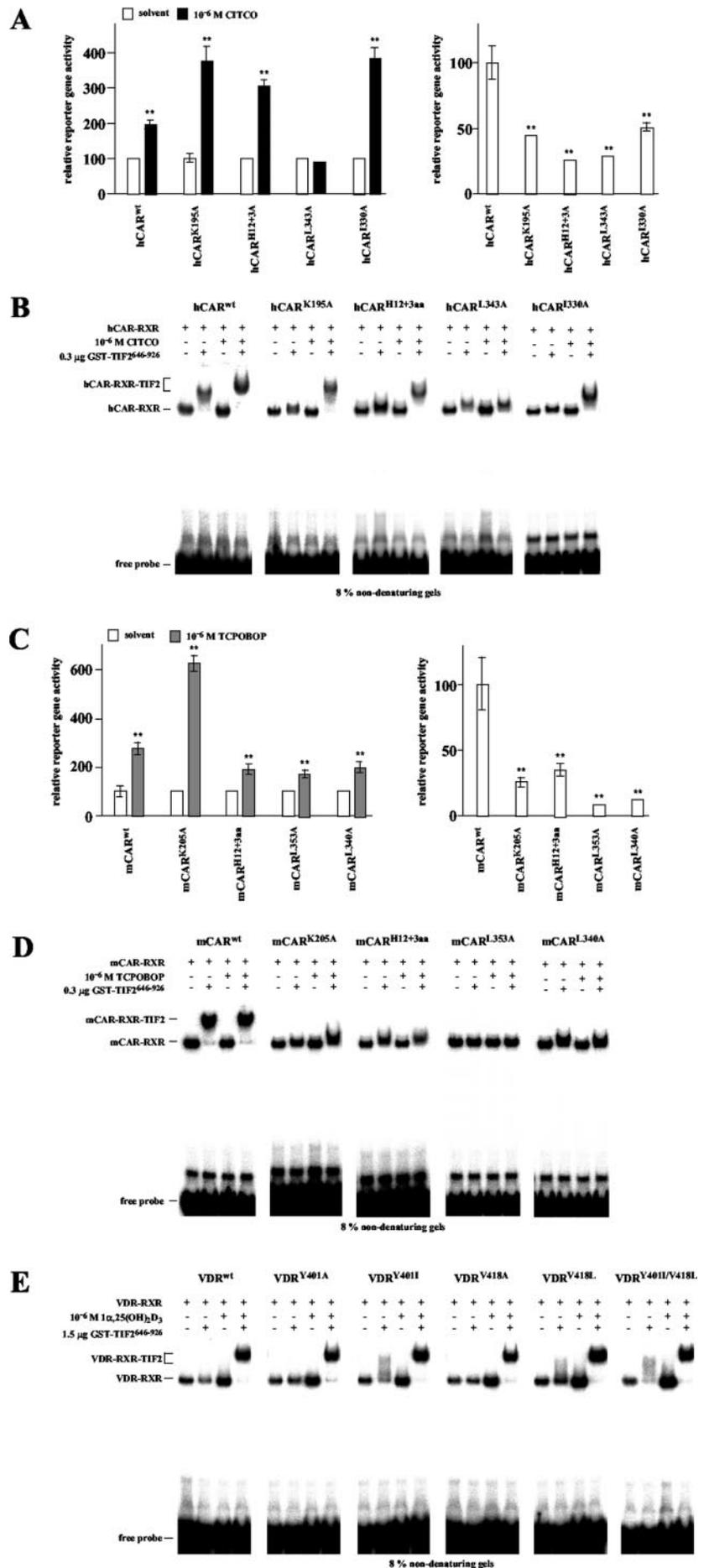


FIG. 3. Stabilizing helix 12 in human and mouse CARs and VDR by interactions with helices 4 and 11. Reporter gene assays were performed with extracts from MCF-7 cells that were transiently transfected with a luciferase reporter construct containing two copies of the DR4-type RE NR1 and expression vectors for wild type (*wt*) and mutated human CAR (*hCAR*) (A) or mouse CAR (*mCAR*) (C). Cells were treated for 16 h with solvent, 1 μ M CITCO (A), or 1 μ M TCPOBOP (C). Data were normalized to the basal activity of the respective receptor mutant (*left panels*). In addition, the basal activity of each mutant was normalized to that of wild type receptor (*right panels*). Columns represent the mean of at least three experiments, and error bars indicate standard deviations. Two-tail, paired Student's *t* test was performed, and *p* values were calculated in reference to the respective solvent control (*left panels*) or the basal activity of the wild type receptor (*right panels*) (**, *p* < 0.01). Supershift experiments were performed with equal amounts of *in vitro* translated wild type and mutated human CAR (B), mouse CAR (D), or human VDR (E) and RXR protein and ³²P-labeled NR1-RE. CAR-RXR and VDR-RXR heterodimers were pre-incubated with solvent, 1 μ M CITCO (B), 1 μ M TCPOBOP (D), or 1 μ M 1 α ,25(OH)₂D₃ (E). Equal amounts of bacterially expressed GST (-) or GST-TIF2₆₄₆₋₉₂₆ (+) were then added. Protein-DNA complexes were resolved from the free probe through 8% non-denaturing polyacrylamide gels. Representative gels are shown.



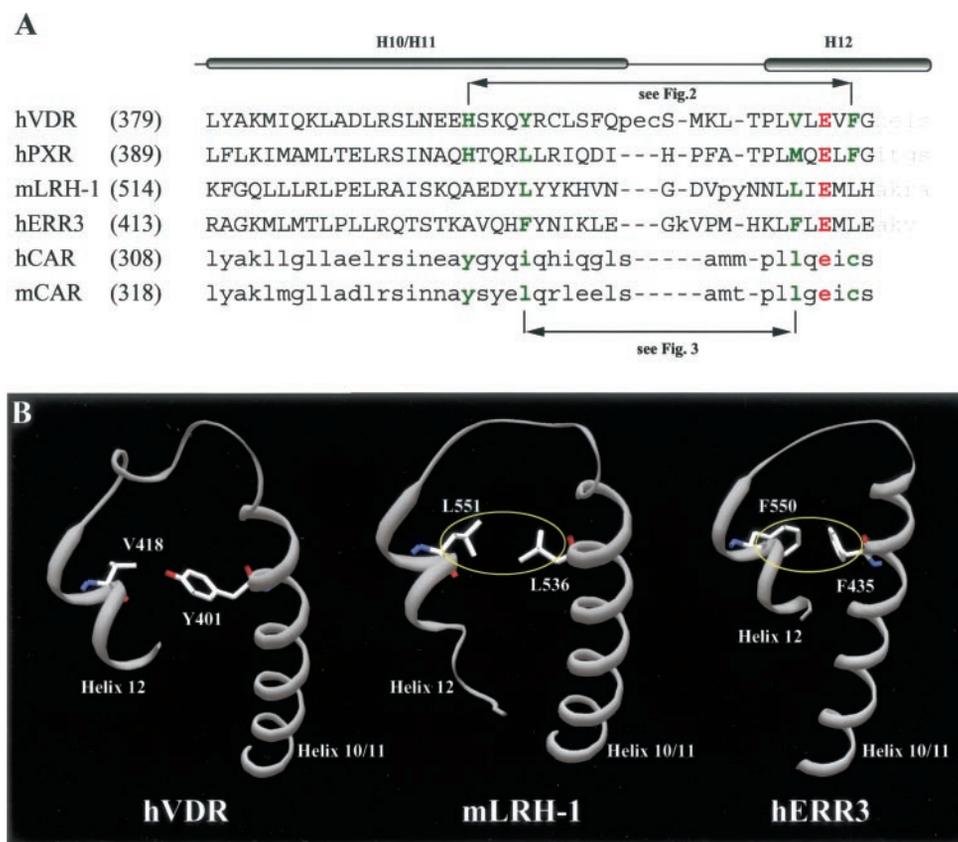


FIG. 4. The structural basis of constitutive activity of NRs. **A**, structural alignment of the C-terminal helices 10/11 and 12 (represented by cylinders) of human VDR (1DB1), human PXR (1NRL), mouse liver receptor homologue 1 (*mLRH-1*) (1PK5), and human estrogen-related receptor γ (*hERR3*) (1KV6) using the vector alignment search tool (VAST) service of NCBI (www.ncbi.nlm.nih.gov). To these four amino acid sequences were aligned the respective regions of human and mouse CARs. *Small black letters* indicate amino acids with uncertain structural position, and *small gray letters* represent amino acids that were missing from the respective crystal structures. **B**, detailed view of the critical amino acids that prevent a hydrophobic interaction between helices 11 and 12 in human VDR but allow it in mouse LRH-1 and human estrogen-related receptor γ . Single letter amino acid abbreviations are used with position numbers. *Red*, negatively charged amino acid; *green*, hydrophobic amino acid.

in vitro interaction of DNA-bound CAR-RXR heterodimers with TIF2 (Fig. 3, A–D). In human CAR, both mutants increased the CITCO-induced transactivation in living cells by a factor of 1.5 to a factor of 1.7, which, due to a reduced basal activity, still resulted in an reduced amount of ligand-induced complex formation with CoA protein (Fig. 3, A and B). In contrast, in mouse CAR even the mutation of Lys-205 increased TCPOBOP-stimulated transactivation by a factor of 2.1, whereas the extension of helix 12 by three amino acids reduced it (Fig. 3C, left panel). Surprisingly, both mutants reduced the *in vitro* interaction with TIF2 (Fig. 3D), which indicates that, at the limiting CoA concentrations that were chosen for this study, the supershift assay may not be sensitive enough to detect the ligand modulation of CAR mutants with low constitutive activity. In summary, in both human and mouse CARs the interaction between the lysine of helix 4 and the C terminus was to be critical for the constitutive activity of the receptor, *i.e.* on the ligand-independent stabilization of helix 12. However, the species-specific CAR agonists CITCO and TCPOBOP differ significantly in their structure and seem to interact differently with the respective ligand-binding pocket. This may explain the different effects of the homologous mutations on the ligand-dependent response of human and mouse CARs.

The structural model of human CAR indicated an additional possibility for the stabilization of helix 12, *i.e.* an interaction between the Lys-343 of helix 12 and the Ile-330 of helix 11 (Fig. 2B). These two amino acids as well as the orthologous amino acids Lys-353 and Lys-340 in mouse CAR (see Fig. 4A) were mutated. In human CAR, L343A blunted the inducibility by

CITCO and reduced the basal activity by 75%, whereas I330A showed a 1.9-fold higher response to ligand than did the wild type receptor and only a 50% reduction of constitutive activity in MCF-7 cells (Fig. 3A). This finding is in accordance with the observed *in vitro* interaction of DNA-bound CAR-RXR heterodimers with TIF2, which is blunted irrespective to the presence of the agonist by the mutation L343A and abrogated only in the absence, but not in the presence, of CITCO with I330A (Fig. 3B). In mouse CAR the orthologous mutations showed a slightly different profile. Both Lys-353 and Lys-340 reduced but did not abrogate TCPOBOP-induced transactivation and reduced the constitutive activity of mouse CAR by 90% (Fig. 3C, right panel). In the supershift assay, both mutants blunted both the ligand-independent and the ligand-dependent interactions of CAR-RXR heterodimers with the CoA protein (Fig. 3D). The response of L353A is in accordance with our previous study on mouse CAR (36). Taken together, both in human and in mouse CAR the Ile-330/Leu-343 and Leu-340/Leu-353 interaction, respectively, showed a significant contribution to the stabilization of helix 12 and, thus, to the constitutive interaction of the receptor with the CoA protein. However, I330A increased the ligand responsiveness of human CAR, whereas L340A decreased that of mouse CAR. This species-specific difference might be due to the different structure of the agonists CITCO and TCPOBOP.

According to a structural alignment (Fig. 4A), the counterparts to Ile-330 and Leu-343 of human CAR are Tyr-401 and Val-418 in human VDR. However, the latter two amino acids are too distant and, also, are not suited for an interaction. This

may explain why the VDR shows no ligand-independent interaction with CoA proteins. However, in the absence of ligand, the mutants Y401I and V418L showed a weak supershift with TIF2, whereas the double-mutant Y401I/V418L displayed a significant ligand-independent interaction with the CoA protein (Fig. 3E). The mutants Y401A and V418A served as controls for the specificity of the gained hydrophobic interaction. In summary, this observation suggests that the endocrine NR VDR has a more flexible and ligand-responsive helix 12 than does the constitutively active orphan NR CAR, because in VDR there is less interaction between helices 11 and 12. To test this hypothesis, we performed a structural alignment of helices 11 and 12 of the receptors VDR and PXR, which are both known for a low constitutive activity and clear ligand responsiveness in comparison with the orphan NR liver receptor homologue 1 (NR5A2) (37) and estrogen-related receptor γ (NR3B3) (38), which are characterized by a high constitutive activity (Fig. 4A). In addition, the respective sequences of human and mouse CARs were aligned. Interestingly, all four constitutively active NRs carry, at both critical positions, large non-polar amino acids that allow a hydrophobic interaction (Fig. 4B), whereas polar and/or small amino acids are found in VDR and PXR.

In this study we demonstrate that, in human CAR, helix 12 is stabilized by three direct amino acid contacts in that helix with partner amino acids in helices 4 and 11 of the LBD. These contacts form the interactions Leu-343/Ile-330, Cys-347/Tyr-326, and that of the C terminus with Lys-195 (Fig. 2B). The charge clamp between Glu-345 and Lys-177 is also important for ligand-independent helix 12 stabilization (Fig. 2A). Interestingly, the ligand-independent stabilization of helix 12 depends largely on Lys-343, because the mutation of this amino acid has the same drastic effect on the constitutive activity of human CAR as does the deletion of the whole helix 12. The same observation was made for the orthologous amino acid in mouse CAR, Leu-353. Surprisingly, the mutation of the partner amino acid of Leu-343, Ile-330 in helix 11, doubled the agonist-induced transactivation of human CAR. This amino acid mutation can be described as a "gain of function." Through this mutation, human CAR behaved more like an endocrine NR with low basal activity and high responsiveness to an agonistic ligand. In the same way, the endocrine NR VDR gains an orphan NR-type behavior when the mutation of the homologous positions in helices 11 and 12 allow a ligand-independent interaction and, therefore, a complex formation with the CoA protein. The mutation K195A in human CAR resulted in a similar shift in the functional profile of the NR as did Ile-330, *i.e.* Lys-195 seems to restrict in the wild type receptor the free mobility of helix 12. Because of the reduced mobility of helix 12, CoA proteins can already interact with CAR in the absence of ligand, such that agonistic ligand binding is not associated with greater amounts of receptor-CoA complex formation and the subsequent dramatic increases in gene activation observed for NRs with low basal activities.

The lack of a common ligand that acts as an agonist for both human and mouse CARs makes a direct comparison of the two orthologous receptors difficult. Moreover, the overexpression of human CAR increases the basal reporter gene activity <4-fold (Fig. 1B), whereas under identical conditions the co-transfection of mouse CAR results in an up to 10-fold increase in constitutive activity (data not shown). Therefore, whereas mutations of mouse CAR can reduce the basal activity by 90%, with human CAR only a maximum reduction of 75% can be observed. Despite these limitations, it appears that the stabilization of helix 12 by a contact between its C terminus and the lysine of helix 4 has the same impact in human and mouse CARs. In addition, the charge clamp between the glutamate in

helix 12 and the lysine in helix 3 is also important for the constitutive activity of both orthologs and is much less critical for the agonist-dependent stabilization of helix 12 in both proteins. However, in mouse CAR, Cys-357 has significantly more impact on the stabilization of helix 12 than does the orthologous position Cys-347 in human CAR. This deficit seems to be compensated by a more dominant role of Ile-330 in human CAR over Leu-340 in mouse CAR, *i.e.* Ile-330 is more efficient than amino acid Cys-347 in controlling the flexibility of helix 12 in the presence of an agonist.

In conclusion, this study has indicated that the constitutive activity of the adoptive orphan NR human CAR is mediated by at least four contacts between amino acids of helix 12 (Leu-343, Glu-345, Cys-347, and the C terminus) and partner amino acids in helices 11 (Tyr-326 and Ile-330), helix 4 (Lys-195), and helix 3 (Lys-177). Mouse CAR uses orthologous amino acid contacts for the same purpose, but the relative impact of each of the interactions is species-specific. Two of the interactions described here, *i.e.* the glutamate-lysine charge clamp and the ligand-induced interaction between helices 11 and 12 (*e.g.* His-397 and Phe-422 in VDR; see Fig. 2), are rather conserved throughout the NR superfamily (see Fig. 4A). However, the two additional interaction possibilities do not seem to be utilized by many other members of the NR superfamily. The ability to use the interaction between the C terminus and helix 4 depends on receptor-specific parameters such as the length of helix 12 and the size of the loop between helices 11 and 12. These requirements make this type of interaction rather specific for CAR. In contrast, the hydrophobic interaction between a pair of large non-polar amino acids in helices 11 and 12 was shown to be the structural basis of the ligand-independent activity of all constitutively active NRs and is not used by the endocrine members of the NR superfamily.

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