Characterization of DNA Complexes Formed by the Nuclear Receptor Constitutive Androstane Receptor*

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The nuclear receptor constitutive androstane receptor (CAR) acts as a xenobiotic sensor and regulates the expression of enzymes, such as several cytochromes P450s and the UDP-glucuronosyltransferase (UGT) type 1A1. CAR binds as a heterodimer with the retinoid X receptor (RXR) to specific DNA sites, called response elements (REs). Clusters of CAR REs, referred to as phenobarbital response enhancer modules (PBREMs), have been identified in several CAR target genes. In this study we confirm that REs formed by direct repeats of two AGTTCA hexamers with 4 spacing nucleotides are optimal for the binding of CAR-RXR heterodimers. In addition, we found that the heterodimers also form complexes on everted repeat-type arrangements with 8 spacing nucleotides. We also observed that CAR is able to bind DNA as a monomer and to interact in this form with different coregulators even in the presence of RXR. Systematic variation of the nucleotides 5′-flanking to both AGTTCA hexamers showed that the dinucleotide sequence modulates the DNA complex formation of CAR monomers and CAR-RXR heterodimer by a factor of up to 20. The highest preference was found for the sequence AG and lowest for CC. The increased DNA affinity of CAR is mediated by the positively charged arginines 90 and 91 located in the carboxyl-terminal extension of the DNA-binding domain of the receptor. Furthermore, we show that one of the three CAR REs of the human UGT1A1 PBREM is exclusively bound by CAR monomers and this is regulated by ligands that bind to this nuclear receptor. This points to a physiological role for CAR monomers. Therefore, both CAR-RXR heterodimers and CAR monomers can contribute to the gene activating function of PBREMs in CAR target genes.

Nuclear receptors (NRs) are a large family of transcription factors (48 human members) that have critical roles in nearly all aspects of vertebrate development and adult physiology by transducing the effects of small, lipophilic compounds into transcriptional response (1). The existence of a highly conserved DNA-binding domain (DBD) and a carboxyl-terminal, structurally conserved ligand-binding domain define the family (2). The ligand-binding domains of most NRs consist of 12 α-helices that form a characteristic, 3-layer sandwich. The most carboxyl-terminal helix, helix 12, contains the activation function 2 domain, which serves as a molecular switch by interacting in the agonistic conformation of the ligand-binding domain with coactivator proteins that activate target gene transcription via further protein-protein interactions (3). NRs can also contribute to gene silencing via the interaction with corepressor proteins that in turn contact histone deacetylases (4). The receptors for estrogen, progesterone, testosterone, cortisol, aldosterone, 1α,25-dihydroxyvitamin D3, thyroid hormone, and all-trans-retinoic acid are classical endocrine NRs (5). However, most of the superfamily members were cloned before their specific ligands were known and were described as orphan NRs (6). Subsequently, some of these compounds, which act as ligands for these orphan NRs, have been identified (1). One of these adopted orphan NRs is the constitutive androstane receptor (CAR; NR1I3 (7)), which has recently been implicated in mediating the effects of xenobiotics on the expression of enzymes, such as cytochrome P450s (8) and UDP-glucuronosyltransferase (UGT) (9). In contrast to classical endocrine NRs that show a very selective ligand binding with Ki values in the order of 1 nM or lower (10), CAR binds a variety of structurally diverse compounds that display a relatively low affinity (Ki in the order of 1 μM) (11). Interestingly, CAR differs from most other NRs by having a strong constitutive activity in the absence of ligand. This can be reduced by the binding of the inverse agonist 5α-androstan-3α-ol or potentiated by the agonists 5β-pregnane-3,20-dione (11) and 6-(4-chlorophenyl)imidazo[2,1-b](1,3)thiazole-5-carbaldehyde o-3,4-dichlorobenzyl-oxime (CITCO) (12).

Nuclear receptor responsive genes are defined through the presence of binding sites for particular NRs, referred to as response elements (REs), in their promoter regions (13, 14). CAR has been shown to form heterodimers with the retinoid X receptor (RXR) on REs that are formed by a direct repeat (DR) of hexameric binding sites (15). Early reports indicated that CAR preferred to bind DR5-type REs (16, 17). However, recently it has been shown that CAR-RXR heterodimers bind optimally to DR4-type REs (8, 18). The closest relatives of CAR, the vitamin D receptor (VDR) and the pregnane X receptor, are also known to bind REs that are formed by two hexameric sites in an everted repeat (ER) arrangement (19–21). A DNA complex formation of CAR for ER binding has not yet been described. The crystal structure of a DNA-bound thyroid hormone receptor (T3R)-RXR heterodimer (22) demonstrated a head-to-tail arrangement of the receptors with the T3R DBD binding to

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‡ The abbreviations used are: NR, nuclear receptor; CAR, constitutive androstane receptor; DBD, DNA-binding domain; DR, direct repeat; ER, everted repeat; GST, glutathione S-transferase; PBREM, phenobarbital response enhancer module; RE, response element; RXR, retinoid X receptor; TIF2, transcription intermediary factor 2; NCoR, nuclear corepressor; T3R, thyroid hormone receptor; UGT, UDP-glucuronosyltransferase; VDR, vitamin D receptor; DBD, DNA-binding domain; DR, direct repeat; DOTAP, N1-[1-(2,3-dioleoyloxypropyl)-N,N,N-trimethylammonium methysulfate.

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the downstream motif and the RXR DBD binding to the upstream motif. Moreover, this study showed that −65% of the DNA contacts of the T₃/R-RXR heterodimer are mediated by T₃/R and explained why this receptor is able to act as a monomer as well (23, 24).

It is inferred that CAR-RXR heterodimers show the same type of DNA binding and polarity as T₃/R-RXR heterodimers (22, 25). However, the investigation of natural CAR responding genes indicated that a single CAR RE seems to 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 response enhancer modules (PbreakM).

The CA) gene contains two DR₄-type REs with an additional binding site for the transcription factor NF-1 (28, the binding motives of the UGT1A1 gene is formed by three CAR REs (9). The authors demonstrated that the three REs of the UGT1A1 PbreakM are necessary for full ligand responsiveness, but how they act together is still unknown.

Although T₃/R preferentially acts as a heterodimer, the T₃/R monomer bound to DNA has been shown to be a fully competent transcription factor (23, 24). Monomer binding in other members of the NR superfamily (e.g. NGRF-B (26) and ROR (27)) appears to be mediated by the Grip box, which is a carboxyl-terminal extension of the DBD (28). This box provides receptor-specific interfaces for an interaction with the nucleotides that flank the 5′ end of the hexameric binding motif. It is known that the DNA binding affinity of T₃/R monomers is strongly modulated by the two nucleotides that directly flank the hexameric binding motif (24). Subsequently, it has been shown that these two 5′-flanking nucleotides also modulate the DNA complex formation of T₃/R-RXR and VDR-RXR heterodimers (29). However, neither the influence of the 5′-flanking nucleotides for CAR-RXR heterodimer binding nor the possibility of CAR monomer binding has been addressed.

In this study, the functional profile of CAR-RXR heterodimers in relation to CAR monomers was investigated. We found that CAR preferentially binds as a heterodimer with RXR to DR4- and ER8-type REs. Surprisingly, we also observed CAR-DNA monomer complexes even in the presence of RXR. CAR monomers and heterodimers showed to interact with co-activator and corepressor proteins. Moreover, we observed that both CAR monomers and CAR-RXR heterodimers have preferences for the dinucleotide that flank the 5′ of the hexameric DNA binding sites. These preferences were mediated by amino acids Arg-90 and Arg-91 of the carboxyl-terminal extension of the DBD. Finally, we indicate a possible physiological role for CAR monomers contributing to the functionality of human UGT1A1 gene promoter PbreakM.

MATERIALS AND METHODS

DNA Constructs

Protein Expression Vectors—Full-length cDNAs for human CAR (16), human RXRs (30), and chicken T₃/R (31) were subcloned into the T₃/SV40 promotor-driven BSGS expression vector (Stratagene, La Jolla, CA). The full-length cDNA for mouse CAR (17) was subcloned into the T₃/cytomegalovirus promotor-driven pCMX expression vector. The point mutants of RXR and human CAR were generated using the QuickChange point mutagenesis kit (Stratagene) and confirmed by sequencing. The helix 12 deletion mutants of RXR and human CAR were created by introducing a stop codon at amino acid positions 442 and 342, respectively, in the proteins. The same constructs were used for both T₃/R and T₃/R-DR4 in vitro transcription/translation of the respective cDNAs and for viral promotor-driven overexpression of the respective proteins in mammalian cells.

Glutathione S-transferase (GST) Fusion Protein Construct—The NR interaction domain of human TIF2, spanning amino acids 1679 to 2453 (33), were subcloned into the GST fusion vector pGEX (Amersham Biosciences).

In Vitro Translation and Bacterial Overexpression of Proteins

Gel Shift and Supershift Assays

The gel shift assays were performed with standard (−10 ng) or the indicated amounts of the appropriate in vitro translated proteins. 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 μg/ml poly[d(C)]C, and 5% glycerol). For supershift experiments 3 μg of bacterially expressed GST, GST-TIF2 (446–926), and GST-NCoR (1679–2453) proteins were added to the reaction mixture. Constant amounts (1 ng) of [32P]-labeled double-stranded oligonucleotides (50,000 cpm) corresponding to one copy of a monomeric or dimeric RE (for core sequences see Figs. 1, 2, and 6) 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× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) and quantified on a Fuji FLA-3000 reader (Tokyo, Japan) using Image Gauge software (Fuji).

GST Pull-down Assays

GST pull-down assay were performed with 50 μl of a 50% Sepharose bead slurry GST-TIF2 (446–926) or GST-NCoR (1679–2453) (pre-blocked with 1 μg/ml bovine serum albumin) and 20 ng in vitro translated γ-S-labeled NRs. Proteins were incubated in immunoprecipitation buffer (20 mM Hepes, pH 7.9, 300 mM KCl, 1 mM EDTA, 4 mM MgCl₂, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 10% glycerol) for 20 min at 30 °C. In vitro translated proteins that were not bound to GST fusion proteins were washed away with immunoprecipitation buffer, GST fusion protein-bound γ-S-labeled NRs were resolved by electrophoresis through 10% SDS-polyacrylamide gels and quantified on a Fuji FLA-3000 reader using Image Gauge software.

Transfection and Luciferase Reporter Gene Assays

MCF-7 human breast cancer cells were seeded into 6-well plates (10⁵ cells/ml) and grown overnight in phenol red-free Dulbecco’s modified Eagle medium, the liposomes were added to the cells. Phenol red-free Dulbecco’s modified Eagle medium is supplemented with 15% charcoal-stripped fetal bovine serum. Plasmid DNA containing liposomes were formed by incubating 1 μg of a reporter plasmid and 1 μg of expression vectors for human CAR with 10 μg of DOTAP (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 medium, the liposomes were added to the cells. Phenol red-free Dulbecco’s modified Eagle medium is supplemented with 500 μl of 15% charcoal-stripped fetal bovine serum was added 4 h after transfection. At this time, either 10 μM 5β-pregn-3,20-dione (Steraloids, Newport, RI), 10 μM CITCO (Biomol, Plymouth Meeting, PA), or 10 μM 5α-androstane-3α-ol (Steraloids) was added. The cells were lysed 16 h after 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).
Molecular Modeling

The high amino acid sequence identity (54.5%) between the DBDs of human T3R/H9252 and human CAR allowed to use the crystal structure of hT3R/H9252-hRXR (Protein Data Bank number 2nll, Ref. 22) as a template for a human CAR-human RXR model. A molecular surface model was produced by the MSMS package (34), which is included in the CHIMERA program package (35). The visual model building was done with Xtalview (36). All the non-conserved amino acid side chains were built up following as close as possible the information of the hT3R/H9252 template.

RESULTS

To determine the multiple DNA complex formation abilities of CAR, gel shift experiments were performed using equal molar quantities of in vitro translated human CAR (hCAR) and human RXR protein and equal amounts of 32P-labeled REs, which are formed of DRs and ERs with increasing numbers of spacing nucleotides (core sequences of the REs are given above). Protein-DNA complexes were resolved from free probe through 8% non-denaturing polyacrylamide gels. Representative gels are shown. The relative amount of protein-complexed DNA was quantified using a Fuji FLA-3000 reader. Columns represent the mean of at least three experiments and bars indicate standard deviation. NS indicates nonspecific complexes.
to both monomeric and dimeric REs. Monomers as well as heterodimers also formed on REs containing AGGTCA motifs (Fig. 2B). These CAR monomer complexes were 10-fold weaker than on REs with AGTTCA motifs (Fig. 2A). Neither CAR homodimer formation nor the independent binding of two CAR monomers was observed on the DR4-type REs. The latter statement was confirmed further by supershift experiments (see Fig. 4B). Taken together, both human and mouse CAR were able to bind to DNA and this was dependent on RXR concentration and hexameric motif sequence.

To compare the monomer and heterodimer formation ability of CAR with other members of the NR superfamily, gel shift experiments were performed with equal amounts of in vitro translated human RXR, human CAR (hCAR), and mouse CAR (mCAR) protein and 32P-labeled REs. The REs were formed either by AGTTCA (A) or by AGGTCA (B) motifs in DR4-type arrangements (left-hand panels) or monomers (right-hand panels), the respective REs core sequences are given above each panel. Protein-DNA complexes were resolved from free probe through 8% non-denaturing polyacrylamide gels. Representative gels are shown. The percentage of protein-complexed DNA was quantified using a Fuji FLA-3000 reader. Columns represent the mean of at least three experiments and bars indicate standard deviations. NS indicates nonspecific complexes.

NRs rely on additional factors to transmit the desired ligand-dependent response to the transcriptional machinery of the cell. To address this functional issue, we investigated the interaction potential of human CAR (and RXR as a control) with the coregulators transcription intermediary factor 2 (TIF2) and nuclear corepressor (NCoR) by GST pull-down assays (Fig. 4A) and supershift assays (Fig. 4B). We also assessed the contribution of helix 12 of CAR and RXR by using respective deletion mutants (ΔH12) of these NRs. In the GST pull-down assay the presence of helix 12 of CAR-RXR heterodimers appeared to prohibit the interaction of the partner receptor with TIF2 or NCoR (Fig. 4A). Some residual interaction with both types of cofactors was observed, when helix 12 domain of RXR was deleted (RXRΔH12). In contrast, wild type CAR displayed comparably high interaction with TIF2 and NCoR. The deletion of helix 12 of CAR (ΔH12) reduced the complex formed with TIF2 (by 30%) but not with NCoR. In the supershift assay all DNA-bound CARwt-RXR heterodimers formed a complex with TIF2 but showed no significant interaction with NCoR (Fig. 4B). Interestingly, the deletion of helix 12 in CAR completely abolished the interaction of DNA-bound CARwt-RXR heterodimers with TIF2, but enabled dominant complex formation with NCoR. Approximately 30% of all DNA-bound CARwt monomers were upshifted by the addition of TIF2, whereas no interaction with NCoR was detectable. In contrast, as a DNA-bound monomer CARΔH12 did not interact with TIF2 but the addition of NCoR completely abolished its complex formation with DNA. It should be noted that all complexes between CAR and TIF2 were obtained in the absence of ligand and that this in vitro protein-protein inter-
action cannot be enhanced greatly by the addition of the human CAR agonist 5β-pregnane-3,20-dione (data not shown). In summary, in solution CAR is able to interact both with TIF2 and NCoR, but when bound to DNA as either heterodimer with RXR or monomer it interacts only with the coactivator. Deletion of helix 12 enables the corepressor to complex with DNA-bound CAR.

To investigate in detail the nature of the CAR monomer DNA complex, we tested whether the sequence 5′-flanking the hexameric DNA binding motif has influence on this interaction. Gel shift experiments were performed with \textit{in vitro} translated human CAR and a series of 16 REs, which represent all possible variations of the 5′-flanking dinucleotides of an AGTTCA motif (Table I, A). The flanking sequence AG (as used in Fig. 2) was found to be optimal for CAR monomer binding, followed by the dinucleotide sequences AA, GA, GG, TA, AT, and TG, which showed 22–45% of maximal monomer binding. The sequences CG and CA mediated only 19 and 16% of maximal binding and the dinucleotides CC, CT, GT, TC, and TT gave less than 10% of the binding relative to the RE with the flanking sequence AG. This demonstrates that the 5′-flanking sequence of the core binding motif modulates monomer binding of CAR by a factor of more than 10. In general, the effects of the variations in front of the downstream motif (Table I, B) were found to be more significant than those in front of the upstream motif (Table I, E). Furthermore, the pattern of CAR-RXR complex formation on the variations in front of the downstream motif (Table I, B) resembled that of CAR monomer binding (Table I, A). This suggests that within a CAR-RXR heterodimer CAR binds to the downstream motif of the DR4-type RE. When we compared the CAR monomer and CAR-RXR heterodimer binding patterns we found only one significant difference. The dinucleotide GC, which is very unfavorable for CAR monomer formation, allows CAR-RXR heterodimer formation at a level greater than the wild type element, when it occurs in front of the downstream motif. Variation of the 3′ position in front of the downstream motif of DR4(T/T) to A, C, or T had no significant effect on the binding of CAR-RXR heterodimers (data not shown) and suggests that analysis of the 5′-flanking dinucleotides is sufficient. On the set of 16 variations of the 5′-flanking sequence in front of the upstream motif we observed that the amount of CAR-RXR heterodimer formation is 3-fold higher, respectively, if the DR4-type RE carries the sequence AG instead of CC in front of the upstream motif. In the model cell line MCF-7 (human breast cancer) the activity of CAR was tested on single copies of the downstream (Table I, C and D) and the upstream (Table I, F and G) motif variations of DR4(T/T). On each of the 31 DR4-type REs the relative basal activity of CAR overexpressing cells (Table I, C and F) was found to correlate well with the relative binding strength of CAR-RXR heterodimers on the same element (Table I, B and E). The pattern of ligand responsiveness of all 30 variations (Table I, D and G) was comparable with that of DR4(T/T), i.e., the human CAR

\[\text{FIG. 3. Comparison of CAR and T,R monomer formation on different types of REs.}\] Gel shift experiments were performed with 10 ng of \textit{in vitro} translated human RXR, human CAR (hCAR), and chicken T3R (cT3R) and \textit{32P}-labeled REs. Equal amounts of proteins were used (see \textit{35S}-labeled proteins above). The REs were formed either by AGTTCA (A) or AGGTCA (B) motifs in DR4-, ER7-, and ER8-type arrangements (compare Figs. 1 and 2). Protein-DNA complexes were resolved from free probe through 8% non-denaturing polyacrylamide gels. Representative gels are shown. The percentage of protein-complexed DNA was quantified using a Fuji FLA-3000 reader. Columns represent the mean of at least three experiments and bars indicate standard deviations. NS indicates nonspecific complexes.
Fig. 4. Interaction of CAR-RXR heterodimers and CAR monomers with cofactors. A, GST pull-down assays were performed with bacterially expressed GST-TIF2-(646–926), GST-NCoR-(1679–2453), and GST alone (as a control) and either wild type or helix 12 deleted (ΔH12) versions of in vitro translated, 35S-labeled human RXR and human CAR (hCAR). After precipitation and washing the samples were electrophoresed through 10% SDS-polyacrylamide gels and the percentage of precipitated NRs with respect to the input amount was quantified using a Fuji FLA-3000 reader. Representative gels are shown. Two-tail, paired Student’s t test was performed and p values were calculated comparing the interaction of the wild type receptors with their respective ΔH12 mutant (*, p < 0.05). B, combined gel shift/supershift experiments were performed with wild type and ΔH12 versions of in vitro translated, human RXR and human CAR and 32P-labeled DR4(T/T). Bacterially expressed GST, GST-TIF2-(646–926), and GST-NCoR-(1679–2453) were used. Protein-DNA complexes were resolved from free probe through 8% non-denaturing polyacrylamide gels. Representative gels are shown. The percentage of protein-complexed DNA was quantified using a Fuji FLA-3000 reader. Columns represent the mean of at least three experiments and bars indicate standard deviations. NS indicates nonspecific complexes.
Table I

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<th>Assay receptor ligand</th>
<th>Gel shift activity</th>
<th>Reporter gene activity</th>
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<td>CAR-RXR heterodimer</td>
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DNA Binding of CAR

16 monomeric REs were derived from the monomer(T)AG, RE, in which the two nucleotides 5' flanking the hexameric binding motif were varied (schematically depicted on top of A). Similarly, 15 dimeric REs were formed on the basis of DR4(T/T) direct repeat RE (schematically depicted on top of B to D), in which the two nucleotides 5' flanking the downstream hexameric binding motif were varied. Gel shift experiments were performed with standardized amounts of in vitro translated human CAR alone (A) or with CAR-RXR heterodimers (B and E) and equal amounts of these 16 monomeric and 31 dimeric 32P-labeled REs. Protein-DNA complexes were resolved from free probe through 5% non-denaturing polyacrylamide gels. The percentage of protein-complexed DNA was quantified using a Fuji FLA-3000 reader.

The percentages of DNA binding and transactivation of CAR monomers and CAR-RXR heterodimers is modulated by the DNA binding sequence AGAGTTCA, which is identical to monomer- and CAR-RXR heterodimer binding sequence AGAGTTCA, respectively. Reporter gene assays were performed with extracts from MCF-7 cells that were transiently transfected with a luciferase reporter construct containing each one copy of the same set of 31 RE variations and the expression vectors for human CAR (C, D, F, and G). The cells were treated with solvent (C and F) or 10 μM 5b-pregnane-3,20-dione (D and G) for 16 h, and relative luciferase activities were measured. The data were normalized to the basal activity of DR4(T/T). Columns A to G indicate the means of at least three experiments and their S.D. Two-tail, paired Student’s t test was performed and p values of the activity of the mutant REs were calculated in reference to the wild type RE (Table I).

A sequence alignment of human CAR with other NRs that are known to bind DNA as monomers, such as NGFI-B (26), ROR (27), and T_R (24), indicated that the positively charged amino acids Arg-90, Arg-91, and Lys-93 of human CAR may mediate the increased affinity of the receptor to DNA. These three amino acids were mutated both individually and simultaneously to alanine. In gel shift experiments (Fig. 5A) and reporter gene assays (Fig. 5B) the activity of equal amounts of the four CAR mutants were compared with that of CARwt. Using the DR4(T/T) as a probe, heterodimer formation with RXR was reduced by 90% with the triple mutant CAR_R90A/R91A/L93A, by 60 and 50% with CAR_R91A and CAR_L93A, respectively, and not at all with CAR_R90A (Fig. 5A). CAR monomers showed a similar profile when binding to the DR4-type RE or to two types of monomer binding sites, i.e. there was no significant influence on DNA binding with CAR_R90A, a substantial reduction with CAR_R91A and CAR_L93A and the most drastic effect with CAR_R90A/R91A/L93A. In MCF-7 cells the functional activity of CARwt in relation to the four mutants was investigated using again DR4(T/T) as a probe (Fig. 5B). Again CAR_R90A showed no difference from CARwt, whereas the activities of CAR_R91A, CAR_L93A, and CAR_R90A/R91A/L93A were reduced by 55, 78, and 90%, respectively. To visualize the location of these hinge domain amino acids, the heterodimer of the DBDs of CAR and RXR was modeled on the basis of the crystal structure of T3R-RXR heterodimers binding to a DR4-type RE (22) (Fig. 5C). In addition, the model structure shows that Gln-94, Arg-97, and Arg-98 are situated at the DNA interacting face of the heterodimer and as a monomer, whereas amino acids Gln-94, Arg-97, and Arg-98 were shown to have only minor impact on the DNA binding of CAR. In confirmation of the experimental results, Lys-93 made no contacts with the DNA. Interestingly, Arg-90 was found to contact the 5' flanking dinucleotide of the downstream motif, whereas Arg-91 interacts with the third base of the hexameric binding motif.

Finally, the physiological role of CAR monomers in relation to CAR-RXR heterodimers was studied using the PBREM of the human UGT1A1 gene as an example. This PBREM contains three CAR binding sites called RE1, RE2, and RE3, respectively (Fig. 6). RE3 contains the optimal CAR monomer binding sequence AGAGTTCA, which is identical to monomer-(T)AG (see Fig. 2A). The functionality of the three natural REs was assessed by gel shift experiments (Fig. 6A) and reporter gene assays (Fig. 6, B and C). On RE1 the ratio between CAR-RXR heterodimer and CAR monomer complex formation was −10:1, on RE2 it was 5:1, and on RE3 only CAR monomers could be detected (Fig. 6A). The latter result is comparable with the profile of CAR binding to monomer-(T)AG (Fig. 2A). In MCF-7 cells the functional, ligand-dependent role of the three
natural REs was tested in relation to DR4(T/T) and monomer-(T)AG (Fig. 6, B and C). On DR4(T/T) CAR showed constitutive activity, which could be potentiated 2.2-fold with the agonist 5β-pregnane-3,20-dione. The constitutive activity of CAR on the other REs was found to be lower, but on the CAR-RXR heterodimer binding site RE1 and on the CAR monomer bind-
ing site RE3 as well as on monomer (T)\textsubscript{Ag} a stimulatory effect by 5b-pregnane-3,20-dione was observed (Fig. 6B). In contrast, no statistically significant ligand effects were found on RE2. The same results were obtained with the newly identified CAR agonist CITCO, which significantly stimulated the activity of CAR on DR4/T/T, monomer (T)\textsubscript{Ag}, RE1, and RE3, but not on RE2 (Fig. 6C). In addition, on DR4/T/T, RE1, and RE3 the inverse agonist 5α-androstane-3β-ol showed a significant activity reduction of CAR. Agonistic and inverse agonistic ligand effects as well as the elevated basal activities were found to be dependent on the overexpression of CAR, i.e. they appear to be specific to this NR. Taken together, the data indicate that RE1 and RE3 of the UGT1A1 PBREM are ligand responsive and appear to function via CAR-RXR heterodimers and CAR monomers, respectively.

DISCUSSION

The selective recognition of discrete sequence elements within promoter regions is a major aspect of specificity in gene regulation. The helical repeat of DNA of −10.5 bp per complete turn predicts that a spacing of 4 or 5 bp between directly repeated hexameric core binding motifs is optimal for placing the DBDs of dimerized NRs to the same side of the DNA. Therefore, the finding of this study, that CAR-RXR heterodimers prefer DR4- and DR5-type REs to other types of DRs, concurs with the theoretical expectations as well as with previously reported results (38). For close relatives of CAR, such as VDR, pregnane X receptor, and TαR, binding to ER-type REs has already been reported (19–21, 39). These receptors were found to prefer ER9- and ER6-type REs, but with lower spacer selectivity than for DR-type REs. In this study, maximal binding of CAR-RXR heterodimers to ER8-type REs was reported, and the broad range of ER5–ER10-type REs that are also recognized by CAR, concurs with the previous reports.

For effective DNA binding, most members of the NR superfamily have to form either homo- or heterodimers. Therefore, the finding that CAR is able to bind as a monomer to DNA is interesting. The monomer binding of CAR is most effective in the absence of RXR and on AGTTCA core binding motifs. Moreover, the monomer binding of CAR to optimized REs is at least as strong as that of TαR on AGGTCA core binding motifs. Whereas both human and mouse CAR bind DNA as monomers, human CAR monomer complexes could be more physiologically meaningful as it shows a stronger preference for this mode of DNA binding than mouse CAR and appears irrespective of the presence of RXR. CAR resembles TαR both in its monomer binding ability as well as in its preference for DR4-type REs (24, 40). In addition, both receptors seem to be comparably broad in their recognition of differently spaced ER-type REs. However, there are differences between these two receptors. For example, TαR is able to form homodimers, and this was not observed for CAR. CAR monomers also have a strong preference for nucleotides 5′-flanking to the hexameric core binding motif. Human CAR clearly prefers the sequence AG to other purine dinucleotide variations. Furthermore, the presence of pyrimidine bases in the 5′-flanking sequence decreases the monomer binding tendency of CAR even more. A variation from AG to CC resulted in the loss of 95% of monomer binding ability and confirms the importance of the 5′-flanking sequence for this mode of DNA binding. Therefore, the octameric sequence AGAGTTCA, but neither AGAGGTC or nor hexamers, should be used for in silico screening of promoter regions for putative CAR monomer action sites.

With the exception of the dinucleotide GC, the variation of the two nucleotides in front of an isolated core binding motif results in the same DNA binding pattern as the variation of the nucleotides in front of the downstream motif of a DR4-type RE. This supports the prediction that within the heterodimeric complex of CAR and RXR, CAR binds to the downstream motif (22). Moreover, the variation of the two nucleotides in front of the upstream motif did not result in more than 3-fold changes in DNA binding of CAR-RXR heterodimers. The simplest explanation for this is that RXR is binding to the upstream motif, so that the observed pattern reflects the 5′-flanking sequence preference of the heterodimeric partner RXR. Interestingly, the dinucleotide AG appears to be preferred by CAR and RXR, whereas GC seems to be most unfavorable. The profile of the in vitro complex formation of CAR-RXR heterodimers translates well into functional activity of CAR in living cells. CAR is most active on the AG and GC variations of the downstream motif and the AG and AT variations of the upstream motif. The ligand responsiveness of CAR in living cells appears to be independent of the DNA binding efficiency because similar -fold inductions are observed.

DNA-bound CAR monomers can interact with coactivators, such as TIF2, and show weak but significant ligand-dependent functional activity in living cells. This indicates that DNA-bound CAR monomers could act as fully functional transcription factors, as has been demonstrated recently for TαR monomers (23). Interestingly, full-length CAR interacts in solution but not on DNA with NCoR. Helix 12 of CAR appears to be crucial for the corepressor-mediated inhibition of DNA binding. An inhibitory role of helix 12 of RXR within heterodimeric complexes has been reported (41), but a similar function of helix 12 of CAR has not yet been described.

Whereas the closest neighbors of CAR, pregnane X receptor and VDR, have no monomer binding potential, we could demonstrate that the positively charged amino acids Arg-90 and Arg-91, but not Lys-93 significantly contribute to the stabilization of CAR on DNA. In this study only mutants of human CAR were investigated, but the sequence identity of human and mouse CAR in this area of the hinge region suggests that the same conclusions could be valid for mouse CAR. It can be assumed that the positively charged arginine pair strongly interacts with the negatively charged phosphate backbone of the DNA, but does not make any base-specific contacts to the 5′-flanking dinucleotide. This suggests that the preference of CAR for the dinucleotide AG is likely not to be a result of direct, base-specific contacts between receptor and DNA, but caused by the geometry of the octameric sequence AGAGTTCA, which seems to be optimal for an interaction with CAR. A similar concept has been suggested for other NRs that bind DNA as monomers (42).

The need of specific sequence 5′-flanking to the core binding motif restricts the number of hexamers (and genes) that may have CAR monomers binding to their promoter region by a factor of at least 10. In addition, one has to consider that most of these putative CAR monomer binding sites are inaccessible to the receptor because they are within a region of condensed chromatin. This reduces the chances that monomeric REs of CAR regulate a gene. Proximity to other transcription factor binding sites that have already caused decondensation of chromatin via the interaction with histone acetyltransferases may increase the potential for a CAR monomer site. Enhancer modules, such as PBREM, are such assemblies of transcription factor binding sites and the most likely places where CAR monomers may have a physiological role. In this study, we have used the PBREM of the human UGT1A1 gene to test this hypothesis. The study by Sugatani et al. (9) has elegantly shown that all three REs of the PBREM of the human UGT1A1 gene are essential for the responsiveness of the gene to CAR (as the mediator of the action phenobarbital). In accordance to our observation Sugatani et al. (9) could detect significant CAR-
FIG. 6. Contribution of CAR heterodimers and monomers to the activity of the UGT1A1 promoter PBREM. The sequence of the PBREM of the UGT1A1 gene with the three CAR binding sites RE1, RE2, and RE3 is indicated above. A, gel shift experiments were performed with in vitro translated human CAR (hCAR) and human RXR (hRXR) protein and 32P-labeled RE1, RE2, and RE3. Protein-DNA complexes were resolved from free probe through 8% non-denaturing polyacrylamide gels. Representative gels are shown. The percentage of protein-complexed DNA (in relation to maximal binding of CAR-RXR heterodimers to RE1) was quantified using a Fuji FLA-3000 reader. NS indicates nonspecific complexes. Reporter gene assays were performed with extracts from MCF-7 cells that were transiently transfected with a luciferase reporter construct containing the indicated dimeric and monomeric REs and an expression vector for human CAR. Cells were treated for 16 h with solvent, 10 μM 5β-pregnane-3,20-dione (B), 10 μM CITCO or 10 μM 5α-androstan-3α-ol (C). Data were normalized to the basal activity of DR4(T/T) without receptor overexpression. Columns represent means of at least three experiments and bars indicate standard deviations. Two-tail, paired Student’s t test was performed and p values were calculated with reference to the respective solvent controls (*, p < 0.05; **, p < 0.01).
RXR heterodimer binding only to RE1 of the enhancer module. We now found that all three REs of the PBREM are capable of binding CAR monomers. RE3 is an exclusive binding site for CAR monomers, because the sequence of its second binding motif is too degenerate to allow RXR binding and heterodimer formation with CAR. Furthermore, RE3 contains the optimal CAR monomer binding sequence AGAGTTCA and shows a CAR binding and transactivation potential that is comparable with isolated monomer(T)AG. In addition, only RE3 and RE1, but not RE2, were found to be responsive to CAR ligands and showed elevated basal activities because of CAR overexpression. This suggests that the PBREM of the \textit{UGT1A1} gene functions via a cooperation of monomeric CAR complexes on RE3 and CAR-RXR complexes on RE1.

In conclusion, this study demonstrates that CAR binds optimally but not exclusively to DR4-type and ER8-type REs. Moreover, CAR was shown to bind as a monomer to DNA and to interact in this form with coactivator proteins. Monomer binding of CAR was found to be optimal on the octameric sequence AGAGTTCA and the arginine pair at positions 90 and 91 is responsible for the increased affinity of CAR to DNA. Most importantly, a physiological role of CAR monomers seems to be a contribution to the function of clustered REs, such as the PBREM of the \textit{UGT1A1} gene.

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Characterization of DNA Complexes Formed by the Nuclear Receptor Constitutive Androstane Receptor

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