Alternative splicing affects the function and tissue-specific expression of the human constitutive androstane receptor

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Abstract

Background: The constitutive androstane receptor (CAR) plays a key role in the control of drug metabolism and transport by mediating the phenobarbital-type induction of many phase I and II drug metabolizing enzymes and drug transporters.

Results: We identified transcripts generated by four different alternative splicing events in the human CAR gene. Two of the corresponding ligand binding domain isoforms demonstrated novel functional properties: First, CAR(SV3), which is encoded by a transcript containing an lengthened exon 7, differentially transactivated target gene promoters. Second, CAR(SV2), which results from the use of an alternative 3' splice site lengthening exon 8, showed ligand-dependent instead of constitutive interaction with coactivators. Furthermore, alternatively spliced transcripts demonstrated a tissue-specific expression pattern. In most tissues, only transcripts generated by alternative splicing within exon 9 were expressed. The encoded variant demonstrated a loss-of-function phenotype. Correct splicing of exon 8 to exon 9 is restricted to only a few tissues, among them liver and small intestine for which CAR function has been demonstrated, and is associated with the induction of CAR expression during differentiation of intestinal cells.

Conclusion: Due to their specific activities, CAR variant proteins SV2 and SV3 may modulate the activity of reference CAR(SV1). Furthermore, we propose that transcriptional activation and regulation of splicing of exon 9 may be coupled to ensure appropriate tissue- and differentiation state-specific expression of transcripts encoding functional CAR protein. Altogether, alternative splicing seems to be of utmost importance for the regulation of CAR expression and function.

Background

The nuclear receptor CAR (NR1I3) plays a pivotal role in the induction of drug metabolism and transport by phenobarbital-type inducers. The hepatic expression of phase I (e.g. CYP2B6, CYP2C9, CYP3A4) and phase II (e.g. UGT1A1, GSTA1) drug metabolizing enzymes and of transporters (e.g. MRP2, SLC21A6) is activated by CAR in response to structurally diverse chemicals [for a review see [1]]. These are represented by the prototypical inducer phenobarbital. More recently, endogenous compounds, like estrogens and bilirubin have been shown to activate CAR [2,3].

CAR is predominantly expressed in liver and in the intestinal epithelium [4,5]. In the non-induced state, the receptor is largely retained in the cytoplasm by interaction with the hsp90 complex, which is mediated by the cytoplasmic CAR retention protein [6]. Treatment with inducers,
among them bilirubin, phenobarbital and acetaminophen [3,7,8], then stimulates nuclear translocation of CAR by a poorly understood mechanism. It is only known that dephosphorylation/phosphorylation steps are involved [7]. Nuclear translocation does not require direct binding of the activator to the receptor. Once translocated to the nucleus, CAR constitutively activates target genes. The receptor binds to DR3, DR4, DR5, ER6 and ER8 motifs of the general nuclear receptor binding site, which have been identified in enhancer and promoter regions of some of the genes regulated [reviewed in [1]]. Usually, CAR binds to DNA as a heterodimer with RXR.

Binding of a ligand is not required for transcriptional activation by CAR. The constitutive activity is explained by the observation that, in contrast to most nuclear receptors, CAR constitutively interacts with coactivators. However, ligand binding may further modulate, induce or inhibit, the transcriptional activity of the receptor. For example, TCPOBOP and CITCO are agonistic ligands of mouse CAR and human CAR, respectively [9,10]. Both chemicals also trigger nuclear translocation [7,10]. On the other hand, androstanediol metabolites have been demonstrated to be inverse agonists of CAR, which inhibit the constitutive activity of the receptor [11].

Alternative splicing, which occurs in up to 60% of human genes [12], has been assumed to be one of the major contributors of protein diversity, as it often results in the expression of protein isoforms. It is also a common phenomenon in the nuclear receptor family. Vitamin D receptor and pregnane X receptor, which are the closest relatives of CAR, exhibit extensive alternative splicing [13,14]. The major CAR transcripts expressed in human and mouse liver are in the size range of 1.3–1.7 kb [4]. One possible explanation of this broad range of transcript sizes may be the existence of alternatively spliced transcripts. In fact, an alternatively spliced CAR transcript, mouse CAR2 has been identified in mouse liver [15]. Mouse CAR2 is characterized by the out-of-frame deletion of exon 8, which inactivates the encoded isoform by C-terminal truncation. Therefore we hypothesized that alternatively spliced transcripts of human CAR may also exist. In this study, we report the identification of four different alternative splicing events in the human CAR gene, which result in up to 12 different transcripts and potentially encoded isoforms. In general, alternative splicing impaired the functional activities of resulting isoforms. However, we identified distinct novel properties of two of them. Furthermore, we demonstrate tissue- and differentiation state-specific alternative splicing of exon 9.

Results

Identification of human CAR splicing variants

To identify human CAR splicing variants, we cloned the cDNA of CAR by PCR using oligo(dT)-primed cDNA of an individual liver and of differentiated Caco-2 cells as templates. The intestinal Caco-2 cells show an induced CAR mRNA expression during enterocytic differentiation (see Fig. 7A). Analyzing the sequences of 23 clones containing the complete open reading frame, we identified the originally published CAR cDNA sequence [4] here referred to as SV1 (8 clones), and the splicing variants SV2 (11 clones), SV4 (3 clones) and SV6 (1 clone) which arise from three different alternative splicing events (Fig. 1A), all resulting in ligand binding domain variant CAR proteins. Two of the splicing events were generated using alternative intronic 3'splice sites, which result in the addition of 12 bp or 15 bp to the 3'end of exon 7 or exon 8, respectively. The encoded protein variants are characterized by the in-frame insertion of 4 amino acids (VSPIT) or 5 amino acids (APYLT), respectively. The third splicing event, skipping of exon 7, results in an in-frame deletion of 39 amino acids. While this work was in progress, splicing variants rising from these three splicing events have been described [16]. By sequencing 10 cDNA clones encompassing exons 8–9, we identified a fourth alternative splicing event, which uses an alternative 3'splice site within exon 9 leading to the out-of-frame deletion of the first 76 bp of the exon. Consequently, the last 42 amino acids of CAR are replaced by 7 new amino acids in proteins encoded by splicing variants containing this out-of-frame deletion (Fig. 1B). If all possible combinations of the four individual alternative splicing events are taken into account, 12 different splicing variants of CAR can be expected which we named SV1-SV12 (Fig. 1A).

All variant proteins of human CAR show a similar intracellular distribution

To analyze the impact of the alterations in the LBD generated by the different splicing events on CAR function, we constructed CAR expression plasmids encoding variant proteins which harbor single changes in the LBD: CAR(SV2), CAR(SV3), CAR(SV4), CAR(SV5) (Fig. 2A). In addition we constructed an expression plasmid encoding CAR(SV6), as we found this particular combination of splicing events in a full length open reading frame cDNA clone, while we did not find any clone containing only the insertion of 12 bp in front of exon 7.

We first investigated whether the intracellular distribution of the variants was altered, compared to reference CAR(SV1). Transient expression of reference CAR(SV1) in COS1 cells demonstrated that the protein was accumulating slightly stronger in the cytoplasm than in the nucleus (Fig. 2B). All CAR protein variants were distributed similarly. The apparent sizes of the variants were in good
Figure 1
Genomic organization and structure of alternatively spliced transcripts of human CAR (A) Genomic organization of the gene (top) and structure of transcripts (bottom). Exons are depicted as open boxes. Introns are shown by horizontal lines. Extension of exons 7 and 8 by use of alternative intronic 3' splice sites is depicted by black boxes. The 5' part of exon 9 which is deleted by use of an alternative 3'splice site within the exon is shown by a gray box with dotted line. Start and stop codons are indicated. TGA* indicates the stop codon of transcripts with the deletion in exon 9. Arrowheads represent the position of the indicated primers (see Table 1). Alternatively spliced transcripts are referred to as SV1 to SV12. (B) Detailed description of the alternative splicing in exon 9. An alternative 3'splice site resides within exon 9. Use of this site results in the out-of-frame deletion of the first 76 bp of the exon. 5' and 3' splice sites are shown in bold lower case letters. Exon sequences represented in the transcripts with the deletion are in upper case letters. Encoded amino acids are shown below. The asterisk denotes the new stop codon.
agreement with the calculated molecular weight of approximately 40 kDa for SV1, SV2, SV3 and SV6 and 35 kDa for SV4 and SV5 (Fig. 2B). Furthermore all protein variants were efficiently and stably expressed in COS1 cells. There was no indication of degradation of particular isoforms. In conclusion these findings indicate that the changes within the LBD of the variant CAR proteins neither influence protein stability nor intracellular distribution.

**CAR protein variants show compromised heterodimerization and DNA binding activities**

To transactivate the expression of its target genes, CAR has to bind to its response elements as a heterodimer with RXRα. Therefore, we analyzed heterodimerization in a mammalian two hybrid assay. Fig. 3A shows that the LBD of reference CAR(SV1) and of CAR(SV3) did interact constitutively with the LBD of RXRα. Remarkably, interaction between RXRα and CAR(SV1) was about 10-fold stronger than interaction between RXRα and CAR(SV3). The LBD of all the other variants failed to interact with RXRα. Treatment with the human CAR-specific agonist CITCO [10] did not influence the interaction of any CAR isoform with RXRα (data not shown).

To investigate DNA binding of CAR variant proteins to response elements in the XREM of CYP3A4 [17] and in the -7.8 kb enhancer of MDR1 (Burk et al., to be published)
Impaired heterodimerization and DNA binding activities of CAR isoforms

(A) Mammalian two hybrid assays were performed in COS1 cells transfected with combinations of an expression plasmid encoding a GAL4-DBD/RXRα–LBD fusion protein and expression plasmids encoding VP16-AD/CAR-LBD fusion proteins of CAR isoforms, as indicated. The columns show the mean activation factors (± S.D.) of the co-transfected GAL4-dependent reporter through interaction of the GAL4-DBD/RXRα–LBD fusion protein with VP16-AD/CAR-LBD fusion proteins of the CAR isoforms SV1 to SV6. The activity of the GAL4-DBD/RXRα–LBD fusion in the presence of empty expression vector pVP16-AD was designated as 1. (B) Gel electrophoretic analysis of [35S]-methionine labeled in vitro synthesized CAR protein isoforms, as indicated. Equal amounts of DNA of the respective expression plasmids were transcribed and translated in vitro and proteins were analyzed, as described in Experimental Procedures. (C) Electrophoretic mobility shift assays were performed using in vitro translated proteins bound to a radiolabeled doublestranded oligonucleotide corresponding to the DR3 motif of the XREM of CYP3A4. Binding reactions contained (+) or lacked (−) the indicated proteins. Complexes of CAR/RXRα heterodimers with the oligonucleotide are marked by an arrow.
Gene-specific transactivation activity of isoform CAR(SV3)

To further analyze the functional activity of the CAR LBD variant proteins, we performed transient co-transfection assays with enhancer/promoter reporter gene constructs of CYP2B6, CYP3A4 and MDR1 in COS1 cells. Co-transfection of reference CAR(SV1) expression plasmid resulted in 11-fold activation of CYP2B6 reporter, 7-fold activation of CYP3A4 reporter and 12-fold activation of MDR1 reporter, thus demonstrating constitutive transcriptional activity of reference CAR(SV1) in COS1 cells (Fig. 4). In contrast, CAR protein variants CAR(SV2), CAR(SV4), CAR(SV5) and CAR(SV6) were not able to transactivate any of the reporter gene constructs significantly. However, CAR(SV3) significantly transactivated the CYP2B6 and MDR1 reporter genes 3-fold and 7-fold, respectively, whereas the CYP3A4 reporter gene was not significantly activated (Fig. 4).

Constitutive and ligand-induced interaction of CAR isoforms with coactivators

CAR demonstrates constitutive interaction with coactivators, which is further enhanced by ligand binding [9]. We analyzed the interaction of CAR isoforms with different coactivators in mammalian two hybrid experiments (Fig. 5). Reference CAR(SV1) showed a strong constitutive interaction with the coactivators tested. The strongest interaction was observed with DRIP205 (100-fold). None of the variant proteins interacted constitutively with coactivators of the p160 family (SRC-1, TIF-2 and ACTR) in this assay. A weak interaction of CAR(SV3) with DRIP205 was observed, whereas the other isoforms did not interact with DRIP205. As expected, the human CAR-specific ligand CITCO enhanced interaction of reference CAR(SV1) with each of the coactivators analyzed. This induction was particularly strong with the coactivators of the p160 family (7- to 10-fold) and only modest with DRIP205 (2-fold). CITCO did not influence the interaction properties of CAR isoforms SV3, SV4, SV5 and SV6. In contrast, interaction of CAR(SV2) with coactivators was strongly induced. The induction was remarkably strong for the interaction with DRIP205 (100-fold), thereby reaching levels of interaction which were obtained with reference CAR(SV1) in the absence of CITCO.

Tissue specific alternative splicing of exons 8 and 9

Besides the very strong expression in liver, mRNA expression of human CAR was reported in heart, muscle, kidney and lung by Northern blot analysis [4]. Consequently, we were interested in the expression pattern of CAR splicing variants in different tissues. As transcript SV2 appeared to represent the most frequent splicing variant among the sequenced CAR cDNA clones, we analyzed the tissue expression pattern of transcripts containing an lengthened exon 8. The assay using primers F1/R1 (Table 1) allowed us to distinguish between transcripts with and without the 15 bp insertion in front of exon 8, represented by fragments of 136 bp and 121 bp respectively (Fig. 6A). Transcripts with the 15 bp insertion were detected in fetal liver, kidney, adult liver, lung, trachea and small intestine (Fig. 6A). In contrast CAR transcripts without this insertion could be detected in every tissue analyzed. RT-PCR with primers F3/R3 (Table 1), targeting exons 2/3 which encode parts of the DBD, confirmed the ubiquitous expression of CAR transcripts in human tissues (data not shown).

As we demonstrated, that most CAR transcripts encode non-functional isoforms, we investigated which tissues may express transcripts encoding functional CAR proteins. RT-PCR with primers F2/R2 (Table 1) allowed to discriminate between transcripts with and without the 76 bp deletion in exon 9, represented by fragments of 135 bp and 211 bp, respectively (Fig. 6B). Only fetal liver, kidney, adult liver, spleen, testis (weakly) and small intestine showed transcripts without this deletion (Fig. 6B). In contrast, transcripts with the deletion in exon 9 were detected in every tissue with the exception of the small intestine. However, analysis of further individual small intestine mucosa samples demonstrated the presence of the 135 bp fragment in varying amounts (data not shown).

Taken together the results of both assays, the ubiquitous expression of CAR transcripts can be attributed to the expression of transcripts with the deletion in exon 9. Thus, transcripts encoding functional CAR protein are not expressed in most tissues. This was confirmed exemplarily by sequencing of lung CAR cDNA clones derived by PCR of the region corresponding to exons 4–9. All clones analyzed, just represented transcript SV5.

Expression and alternative splicing of CAR during intestinal differentiation of Caco-2 cells

Fig. 7A shows that CAR expression cannot be detected by Northern blot in intestinal cell lines including subconfluent and confluent Caco-2 TC7. However, if the latter cells were grown for 15 days post confluence, a very strong expression of CAR was observed. As enterocytic differentiation of Caco-2 TC7 cells took place when the cell culture reached confluence [18], we assumed that CAR expression...
**Figure 4**

**CAR(SV3) differentially transactivates promoter reporter genes** COS1 cells were co-transfected with enhancer/promoter reporter gene plasmids and expression plasmids encoding CAR isoforms, as indicated. The columns show the mean activation factors (± S.D.) of the respective reporter genes by the indicated CAR isoforms. The activity of each reporter in the presence of empty expression vector pcDNA3 was designated as 1. Statistically significant differences are indicated by asterisks (***, p < 0.001).
Constitutive and ligand-dependent coactivator interactions of CAR isoforms

Mammalian two hybrid assays were performed in COS1 cells transiently transfected with combinations of expression plasmids encoding VP16-AD/CAR-LBD fusion proteins and GAL4-DBD/coactivator-RID fusion proteins, as indicated, together with the reporter gene plasmid pGL3-G5. The columns show the mean activation factors (± S.D.) of the GAL4-dependent reporter through interaction of GAL4-DBD/coactivator-RID fusion proteins (SRC-1, TIF-2, ACTR, DRIP205) with VP16-AD/CAR-LBD fusion proteins of the CAR isoforms SV1 to SV6. Open and filled columns indicate treatment with vehicle Me2SO and CITCO (1 µM), respectively. The activity of each GAL4-DBD/coactivator-RID fusion in the presence of the empty expression vector pVP16-AD (VP16) treated with Me2SO was designated as 1.
was induced by differentiation. Enterocytic differentiation of post-confluent Caco-2 cells was confirmed by analyzing the expression of the intestinal differentiation marker genes sucrase-isomaltase and alkaline phosphatase. Expression of both genes was strongly induced by post-confluent growth (data not shown). Since we could demonstrate tissue-specific alternative splicing of CAR, it was of interest to see whether alternative splicing may also be associated with the induction of CAR expression by differentiation. RT-PCR with primers F2/R2 clearly demonstrated that confluent, undifferentiated Caco-2 cells predominantly express transcripts with the deletion in exon 9. However, Caco-2 cells grown for 15 days post confluence showed a strong expression of transcripts without and a markedly reduced expression of transcript with the deletion in exon 9 (Fig. 7B). Thus, the strong induction of CAR expression by enterocytic differentiation was also associated with a switch in alternative splicing.

Discussion

We here describe the identification of alternatively spliced transcripts of the human CAR gene which result from four different splicing events and present a comprehensive functional characterization of encoded isoforms. While this work was in progress, isoforms generated by three of these splicing events have been described [16,19]. In addition to these, we here describe for the first time the identification of transcripts generated by use of an alternative 3' splice site within exon 9. The encoded C-terminally truncated proteins (e.g. CAR(SV5)) are characterized by loss of α-helices H10/11 and H12 of the LBD. H12 encodes the AF2 domain, which is essential for ligand-dependent transactivation of nuclear receptors in general and also for the constitutive transactivation activity of CAR [20]. In mouse CAR, the out-of-frame deletion of exon 8 has similar structural consequences, resulting in the replacement of the last 78 amino acids by 6 new residues [15].

Compared to reference CAR(SV1), the intracellular distribution of the variants was not altered. Unexpectedly, even CAR(SV5) which lacks the last 42 residues did not show decreased nuclear localization. Loss of these C-terminal amino acids also removes the xenochemical response signal (residues 313–319) of CAR, which is essential for inducer-dependent nuclear translocation in hepatocytes as well as for constitutive nuclear localization in HEK293 cells [21]. The different result obtained with CAR(SV5) in COS-1 cells may be due to a specific post-translational modification which alters functional properties of the variant. One possibility may be phosphorylation, as the tripeptide TSR representing the first three new C-terminal residues of CAR(SV5) fits to the consensus phosphorylation site recognized by protein kinase C.

We showed that none of the CAR protein variants bind to DNA as a heterodimer with RXRα. Our data are in agreement with the strongly impaired DNA binding activity observed recently for the isoforms we here call SV2, SV3, SV4 and SV6 [16]. The missing DNA binding activity of CAR(SV5) is in agreement with the observation that the structural similar mouse CAR2 also did not bind to DNA [15]. Most likely, this can be attributed to the lack of helix H10/11 in CAR(SV5), as this helix participates in the heterodimerization interface [22]. Altogether, impaired interaction with RXRα seems to be the mechanism underlying the loss of DNA binding of the CAR isoforms.

In agreement with their impaired DNA binding, CAR protein variants SV2, SV4, SV5 and SV6 did not transactivate significantly either reporter gene construct tested. In contrast, CAR(SV3) transactivated the CYP2B6 and MDR1 reporter constructs with 30% and 50% of the activity of reference CAR(SV1), respectively. However, the isoform did not activate CYP3A4 significantly. We conclude that CAR(SV3) may differentially regulate CAR target genes. Furthermore, CAR(SV3) transactivation activity and interaction with RXRα imply that this variant most likely can

| Primer | Direction | Sequence (5’-3’) | Exon | Detected variants/Fragment size |
|--------|-----------|-----------------|------|-------------------------------|
| F1     | forward   | TGG AGT TGC TCT TTC ACT TCC | 7    | SV 1, 3, 5, 9/121 bp          |
| R1     | reverse   | TCT CTC TGG GTA ACT CCA GGT C | 8    | SV 2, 6, 8, 10/136 bp         |
| F2     | forward   | GAC CTG GAG TTA CCC AGA GAG A | 8    | SV 1, 2, 3, 4, 6, 7/211 bp    |
| R2     | reverse   | ATC TCC TGG AGC AGC GGC ATC AT | 9    | SV 5, 8, 9, 10, 11, 12/135 bp |
| F3     | forward   | CTA CCA CTT TAA TGC GCT GAC T | 2    | SV 1–12 (all variants)/121 bp |
| R3     | reverse   | CCT CTG AGT CTT GCT GAC TTC | 3    |                                |

Table 1: Oligonucleotides used for RT-PCR analysis
bind to DNA in a cellular context, in contrast to the impaired DNA binding seen in vitro. CYP2B6 and MDR1 have in common, that in both genes exclusively DR4-type nuclear receptor binding sites mediate the activation by CAR (ref. [28], and Burk et al., to be published elsewhere). In contrast, ER6- and DR3-type binding sites mediate activation of CYP3A4 [17]. The difference in the type of binding site may explain the observed gene-specific transactivation activity of CAR(SV3).

CAR constitutively interacts with coactivators SRC-1, TIF-2 and ACTR [11,23,24]. Using mammalian two-hybrid assays, we here show for the first time that reference CAR(SV1) also interacts constitutively with DRIP205. Recently, it has been reported that constitutive activity of CAR results from a few unique structural features which mimic the function of ligand with respect to reorientation of H12 into an active conformation [20]. The insertion in CAR(SV2) is not targeting the respective regions of the CAR protein, it alters the loop connecting H8 and H9. A structure-based sequence alignment showed that length and structural features of the H8/H9 loop are highly conserved among nuclear receptors. Furthermore, residues of that loop also participate in the heterodimerization interface [22]. Interestingly, heterodimerization is also required for full coactivator recruitment [20]. Thus we suspect that impaired heterodimerization with RXRα causes the loss of constitutive coactivator interaction of CAR(SV2) and of the other isoforms, as they all are compromised in heterodimerization with RXRα. In contrast, GST pull-down experiments have shown that CAR protein variants SV2, SV3, SV4 and SV6 exhibited specific interactions with SRC-1 [16]. These variants may therefore retain the capability to interact with coactivators in vitro. However in the more physiological situation of a cell-based assay, interaction cannot be detected.

The molecular modeling analysis of the human CAR LBD revealed that the structure of the ligand binding pocket is probably not altered by the 5 amino acid insert present in CAR(SV2). The insertion of 4 amino acids and the deletion of 39 amino acids in CAR(SV3) and CAR(SV4), respectively, are predicted to alter the structure of the ligand binding pocket [16]. Likewise CAR(SV5) is probably
**Figure 7**
CAR expression and alternative splicing of exon 9 during enterocytic differentiation of Caco-2 TC7 cells. (A) Northern Blot analysis with polyadenylated RNA of the indicated cell lines. Caco-2 TC7 cells were analyzed from subconfluent (sub), confluent (confl) and 15 days post-confluent (15d pc) cultures. The blot was sequentially hybridized with probes for the genes indicated. The arrow marks the major CAR mRNA species of 1.4 to 1.7 kb. (B) Analysis of the expression of transcripts with alternatively spliced exon 9 by qualitative RT-PCR with random hexamer primed cDNA of polyadenylated RNA of Caco-2 TC7 cells cultured until confluence (confl) and for 15 days post-confluent (15d pc). PCR was performed with primers F2/R2 (Table 1). SV1 and SV5 denote control reactions performed with DNA of the corresponding CAR isoform expression plasmids. The lane on the left shows a 50 bp ladder size marker. By mixing DNA of SV1 and SV5 CAR isoform expression plasmids in different molar ratios, we confirmed that both fragments were amplified with equal efficiency (data not shown).
impaired in ligand binding, as homology modeling of the CAR LBD has shown that residues of helices H10/11 and H12 should participate in the ligand binding cavity [20]. Thus we assume that CAR(SV2) is the only variant capable of ligand binding. Indirect evidence for this assumption is provided by the observation that among all variants human CAR-specific ligand CITCO solely induced the interaction of CAR(SV2) with coactivators. The insert in CAR(SV2) thus reverts the constitutive coactivator interaction to a ligand-dependent one. Having bound a ligand seems to induce a sufficiently stabilized active conformation so that heterodimerization with RXRρ is no longer required. Altogether ligand-dependent coactivator interaction suggests a possible functional role for CAR(SV2). The isoform may sequester coactivators of reference CAR(SV1) and consequently modulate the activity of the latter. Thus the ratio of CAR(SV2) and reference CAR(SV1) may determine CAR-dependent activation by inducers which also act as ligands.

Alternative splicing of CAR is obviously regulated in a tissue- and differentiation state-specific manner. Splicing which leads to a lengthened exon 8 occurs in only a few tissues. In most cases these are the tissues which express transcripts with correctly spliced exon 9. However both sets are not completely congruent, thus suggesting an independent regulation of these two splicing events. Transcripts with the deletion in exon 9 are ubiquitously and constitutively expressed and appear to be the only ones expressed in most tissues. The constitutive expression of these transcripts will be very low in general, as in many tissues and cell lines Northern blot analysis did not detect any CAR expression or only a very weak one (ref. [4], and Fig. 8A). Thus, exclusive use of the alternative 3′ splice site within exon 9 appears to be associated with low expression. Correct splicing of exon 9, which is a prerequisite of functional CAR expression, is restricted to only a few tissues, among them small intestine and liver where CAR function has been demonstrated [5,25]. A functional role of CAR in the other tissues which express transcripts with correctly spliced exon 9 has not yet been demonstrated.

Interestingly, we observed a switch from use of the alternative 3′ splice site within exon 9 towards use of the correct site during enterocytic differentiation of intestinal Caco-2 cells. Simultaneously, CAR expression was strongly induced. This raises the intriguing question, whether transcription and splicing of CAR could be coordinately regulated. Recently, it has been shown that activated steroid receptors simultaneously regulate transcription and splicing most likely through the recruitment of co-regulators involved in both processes [26]. Interestingly, expression of CAR is induced by hormone-activated GR, which binds to a glucocorticoid response element in the promoter region of the gene [27]. We propose that the

tissue- and differentiation state-specific correct splicing of exon 8 to exon 9 may be a transcription-coupled mechanism to restrict expression of functional CAR to the relevant target sites. The low expression which is seen ubiquitously appears to be associated with the use of the alternative 3′ splice site within exon 9, thus ensuring that no functional transcripts are produced ectopically. This may represent a mechanism compensating for inappropriate transcription of the CAR promoter.

Conclusions
In general, the LBD isoforms of CAR, which result from alternative splicing, demonstrate a loss of function phenotype. However, and in contrast to recent studies [16,19] we here clearly have shown that two variants retain distinct functional activities and may therefore exert specific functions. Furthermore, alternative splicing within exon 9 appears to be functionally significant, as it seems to be involved in the control of the tissue- and differentiation state-specific expression of functional CAR. Thus alternative splicing of CAR further adds to the complex regulation of CAR expression and function.

Methods
Materials
All cell culture media, supplements and fetal calf serum were obtained from Invitrogen (Carlsbad, CA). 6-(4-chlorophenyl)imidazo [2,1-b]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) was obtained from Biomol (Plymouth, PA).

Cloning of CAR splicing variants by PCR and construction of CAR expression plasmids
The open reading frame of CAR (bases 158–1205 of Genbank accession number NM_005122 / GI 32189358) was amplified by PCR from oligo(dT) primed cDNA samples of an individual human liver and differentiated Caco-2 cells with primers 5′-ATG AAT TCC ACC ATG GCC AGT-3′ and 5′-AAT AAG CTT TCC CAC TCC GAG TTA CCC-3′ and 5′-CGT CTA GAT TAG CTT GAG ATC TCC TGG AGC AG-3′, which introduced an XbaI site and modified the stop codon to TAA. PCR products of approximately 1000–1100 bp were purified and digested with EcoRI and XbaI. The digested fragment was cloned into appropriately digested pCDNA3 vector (Invitrogen) and the resulting clones were sequenced. Thus we obtained eukaryotic expression plasmids (pCdhCAR) for transcripts SV1, SV2, SV4 and SV6. Part of exons 8–9 (bases 970–1266) was amplified by PCR from oligo(dT)-primed cDNA of a human liver with primers 5′-AAT GAA TTC ACC GAC CTG TTA CCC-3′ and 5′-AAT AAG CTT TCC CAC TCC AGT GTA TCC-3′. The oligonucleotides introduced an EcoRI and a HindIII restriction site on the 5′- and 3′-ends of the amplified fragment, respectively. PCR fragments of
200–300 bp were purified, digested with EcoRI and HindIII and subsequently cloned into appropriately digested vector pGEM-7Zf(+) (Promega, Madison, WI). Resulting clones were sequenced.

The eukaryotic expression plasmid for CAR(SV5) was constructed by cloning the 910 bp EcoRI/Xmal insert fragment of pcDhCAR(SV1) and the 130 bp Xmal/HindIII insert fragment of a pGEM exon 8–9 clone (see above) containing the deletion in exon 9 into appropriately digested vector pcDNA3.1(−) (Invitrogen). The internal Xmal site resides at position 1067 of the CAR cDNA. To construct a eukaryotic expression plasmid for transcript SV3, the 15 bp insert lengthening exon 8 was deleted from pcDhCAR(SV6) by use of the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the recommendations of the manufacturer. The introduction of the deletion and the absence of other undesired mutations were verified by sequencing.

**Other plasmid constructs**

The regions encoding the LBD (amino acids 105–348 of the reference sequence) of CAR protein variants SV1, SV2, SV3, SV4 and SV6 were amplified by PCR with primers 5′-TTA GAA TTA TTC CGT CTT AAG GAG CAA GAA GAG-3′ and 5′-TTA TCT AGA CTA AGT CAT TTG GTG CCG CGC C-3′, using the respective expression plasmids as templates. The region encoding the LBD of CAR(SV5) was amplified by PCR with the same forward primer and reverse primer 5′-TTA TCT AGA GCT GCA GAT CTC CTG GAG C-3′, 5′-TTA GAA TTC CAA CTG AGT AAG GAG CAA GAA GAG-3′, using the respective expression plasmids as templates. PCR fragments were cloned into appropriately digested vector pVP16 (BD Biosciences Clontech, Palo Alto, CA), thus generating fusion proteins of the VP16 activation domain and the respective CAR LBD. All constructs were verified by sequencing.

The sequence encoding the LBD of human RXRα (amino acids 226–462) was amplified by PCR using 5′-TTA GAA TTC GCC AAC GAG GAC ATG CCG-3′, which introduced an EcoRI site and 5′-TTA TCT AGA AGT CAT TTG GTG CGG CGC C-3′, which introduced an Xmal site. The resulting PCR fragment was digested with EcoRI and Xmal and cloned into appropriately digested vector pM (BD Biosciences Clontech) with 100 units/ml penicillin and 100 mg/ml streptomycin, 2 mM L-glutamine, and 10% fetal calf serum. The human colon carcinoma cell lines LS174T and LS180 were obtained from the American Type Culture Collection (Manassas, VA). The human colon carcinoma cell line Caco-2 TC7 [31] and the human small intestinal cell line BN [32] were kindly provided by U. Meyer (Division of Pharmacology/Neurobiology, Biozentrum of the University of Basel, Switzerland) and G. Pang (Royal Newcastle Hospital, University of Newcastle, Australia), respectively. The human hepatoblastoma cell line HepG2 was obtained from B. Sperker (Institute of Pharmacology, University of Greifswald, Germany). LS174T, LS180, Caco-2 TC7 and BN were cultivated in Dulbecco's modified Eagle medium buffered with 25 mM HEPES, supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin, 2 mM L-glutamine, and 10% fetal calf serum. HepG2 cells were grown in Minimal Essential Medium, supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin, 2 mM L-glutamine and 10% fetal calf serum. Cells were grown at 37°C and 5% CO2 in a humidified incubator.

For differentiation experiments, Caco-2 TC7 cells were plated on collagen I coated dishes (BD Biosciences Discovery Labware, Bedford, MA) and grown in standard growth medium until confluence was reached. Then, the medium was replaced by differentiation medium, containing 5% fetal calf serum and culture was continued for 15 days with daily exchange of differentiation medium.

**Transient transfections, mammalian two-hybrid and reporter gene assays**

One day before transfection, COS1 cells were plated in 24-well plates (BD Biosciences Discovery Labware) at a den-
sity of 3 \times 10^4 \text{ cells/well. Transient transfections were performed as described} [33]. For Two-Hybrid assays, cells were transfected with 110 ng of the reporter gene plasmid pGL3-G5, 10 ng of expression plasmids encoding GAL4-DBD/RXRα-LBD or GAL4-DBD/coactivator-RID fusions, 80 ng of expression plasmids encoding the respective VP16-AD/CAR-LBD fusions and 20 ng of β-galactosidase reference plasmid pCMVβ (BD Biosciences Clontech) per well. In some experiments, cells were subsequently treated with 1 μM C3TICO dissolved in Me2SO or with an equivalent amount (0.1%) of Me2SO only. Luciferase and β-galactosidase activities were analyzed as described [33]. To identify statistically significant differences, one-way analysis of variance with Student-Newman-Keuls post test was performed with the mean values of at least three independent experiments done in triplicates using GraphPad InStat version 3.05 for Windows 95 (GraphPad Software, San Diego, CA).

**Electrophoretic mobility shift assays**

Electrophoretic mobility shift assays were performed as previously described [30]. Human CAR isoforms and RXRα protein were synthesized using the CAR isoform expression plasmids and pCMX-hRXRα (kindly provided by R. Schüle, Klinik für Tumorbiologie, University of Freiburg, Germany), respectively and the TNT T7 Quick Coupled transcription/translation system (Promega). Oligonucleotides for the CYP3A4 DR3-RE3 motif were as follows: sense, 5’-GAT CCG CAG AGG GTC AGC AAG TTC ATT CAG A-3’; antisense, 5’-GAT CTC TGA ATG AAC TTG CTG ACC CTC TGC G-3’. Retarded complexes were quantified with the BAS1800 II phosphor-storage scanner (Fuji, Kanagawa, Japan) and AIDA software (Raytest, Straubenhardt, Germany).

**Protein analysis**

For Western blot analysis of CAR isoforms, COS1 cells were co-transfected with the respective expression plasmids and β-galactosidase plasmid pCMVβ (Clontech) using calcium phosphate co-precipitation as described [30]. Cells were harvested 24 hours after transfection and cell pellets were resuspended in 10 mM Tris-Cl pH 7.8, 5mM KCl, 2mM MgCl2. Cell membranes were lysed by addition of Nonidet P-40 to 0.5%, and nuclei were collected by centrifugation. The cytoplasmic proteins in the supernatant were precipitated with acetone. Nuclei and precipitated cytoplasmic proteins were boiled in 62.5 mm Tris-Cl pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 0.02% bromphenol blue and protein samples were resolved on a sodium dodecyl sulfate-10% polyacrylamide protein gel. Western blotting was performed as described [29]. CAR proteins were detected with a CAR-specific polyclonal antibody [7], kindly provided by M. Negishi (National Institute of Environmental Health Sciences, Research Triangle Park, NC). Immuno-reactive bands were visualized using the chemiluminescence substrate SuperSignal West Dura Extended Duration (Pierce, St. Augustine, Germany) and the digital CCD-camera LAS-1000 (Fuji). Results were analyzed with AIDA software (Raytest).

CAR proteins were labeled with [35S] methionine by in vitro synthesis using the TNT T7 Quick Coupled transcription/translation system (Promega) and separated on a sodium dodecyl sulfate-10% polyacrylamide protein gel. The gel was stained with Coomassie Brilliant Blue, incubated in 0.5 M sodium salicylate and dried. The dried gel was exposed to phosphor-storage imaging plates and quantified with the BAS 1800 II phosphor-storage scanner (Fuji) and AIDA software (Raytest).

**Isolation of total RNA and RT-PCR**

Total RNA was prepared from human liver samples and small intestine and colon mucosa samples using the RNasy Mini Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. The origin of the human liver and intestine samples has been described previously [33]. RNA was treated with DNase I to remove contaminating genomic DNA.

5 μg total or polyadenylated RNA was reverse-transcribed with Superscript II reverse transcriptase (Invitrogen) with random hexamer primers according to the standard protocol of the manufacturer.

Expression of CAR transcripts was analyzed by PCR with the primers listed in Table 1. PCR was performed with cDNA corresponding to 500 ng RNA, 1x PCR buffer with 1.5 mM MgCl2 (Roche Diagnostics, Mannheim, Germany), 200 μM each of dATP, dCTP, dGTP and dTTP, 1 μM each of the forward and reverse primers, and 1.25 U Taq polymerase (Roche Diagnostics). After an initial denaturation step at 94°C for 2 min, cycling conditions comprised 30 cycles of 94°C for 30 sec, 65°C for 30 sec, and 72°C for 30 sec, followed by a final extension step of 72°C for 5 min. PCR products were separated on 10% polyacrylamide gels and visualized by staining with ethidium bromide.

**Northern blotting**

Preparation of polyadenylated RNA and Northern blot analysis were done as described previously [30]. Radioactive probes were synthesized using the DECAprime II Kit (Ambion, Austin, TX). To synthesize a probe specific for human CAR, the cDNA fragment of pcDHCAR(SV1) was used. The origin of the probe specific for human GAPDH has been described [30].
Abbreviations
CAR, constitutive androstane receptor; CYP, cytochrome P450; DR, direct repeat; ER, everted repeat; TCPOBOP, 4-bis [2-(3,5-dichloro-pyridyloxy)]benzene; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b]thiazole-5-carbaldehydeO(3,4-dichlorobenzyl)oxime; LBD, ligand binding domain; RXRα, retinoid X receptor α; RID, receptor interaction domain; SRC-1, steroid receptor coactivator 1; TIF-2, transcriptional intermediary factor 2; ACTR, activator of thyroid hormone and retinoic acid receptors; DRIP205, Vitamin D receptor interacting protein 205; MDR, multidrug resistance; DBD, DNA binding domain; AD, activation domain; XREM, xenobiotic-responsive enhancer module; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; kDa, kilo Dalton; bp, base pairs.

Competing interests
None declared.

Authors’ contributions
K.A.A. and O.B. identified the splice variants of CAR. K.A.A. carried out the experiments to functionally characterize CAR variant proteins and to analyze expression of CAR transcripts in human tissues and drafted the manuscript. M.E. participated in preparation of the manuscript. O.B. conceived of and directed the study, carried out the studies of CAR expression in human cell lines and prepared the manuscript. All authors read and approved the final manuscript.

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