Coordinate Regulation of Cytochrome P450 1A1 Expression in Mouse Liver by the Aryl Hydrocarbon Receptor and the β-Catenin Pathway

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The expression of cytochrome P450 (CYP) 1a1 and other drug-metabolizing enzymes is controlled by the aryl hydrocarbon receptor (AhR), which is activated by dioxin-type inducers leading to transcriptional induction of target genes. Here, we show that a second level of transcriptional control exists in hepatocytes, which is tightly linked to the Wnt/β-catenin/T-cell factor (TCF) signaling pathway. In transgenic mice, hepatic expression of CYP1A1 (and other CYP isoforms) is stimulated by the expression of mutationaly activated β-cateninS33Y in the absence of AhR-activating compounds but repressed after knockout of β-catenin. These effects were further analyzed in vitro, and the stimulatory role of β-catenin was ascribed to a TCF-binding site within the CYP1A1 promoter. Moreover, β-catenin signaling acted cooperatively with AhR agonists via AhR-binding sites on the DNA during the induction of Cyp1a1 in vivo and in vitro. Activation of β-catenin enhanced the transactivation potential of ligand-activated AhR at its DNA-binding sites without altering the total amount of DNA-bound AhR. Coimmunoprecipitation demonstrated a physical interaction between AhR and β-catenin. Furthermore, the present results suggest that transcriptional induction of the AhR by β-catenin does not play a major role in β-catenin-dependent regulation of Cyp1a1 expression and that inhibition of β-catenin signaling by ligand-activated AhR, as recently observed in the intestine does not occur in mouse liver. In conclusion, signaling through β-catenin activates basal CYP1A1 expression and augments CYP1A1 induction by AhR ligands through enhancement of the transactivation potential of the AhR.

Key Words: metabolic zonation; drug metabolism; enzyme induction; Wnt signaling; dioxin; XRE.

Cytochrome P450 (CYP) monooxynogenases are important players in the metabolism of exogenous and endogenous compounds. The majority of CYPs from subfamilies 1–3 exhibit broad substrate specificities and catalyze the metabolism of drugs, environmental pollutants, and carcinogens. Transcriptional induction of CYP expression following the exposure to xenobiotics has been studied extensively. Several cytosolic xenobiotic-sensing receptors were characterized which act as transcription factors in the nucleus after ligand activation, as reviewed by Xu et al. (2005). CYP1 family members are mainly regulated by the aryl hydrocarbon receptor (AhR) (Bock, 1994): Upon stimulation by planar polycyclic aromatic hydrocarbons, e.g., 2,3,7,8-tetrachloro-p-dibenzofuranin (TCDD), the AhR translocates to the nucleus, dimerizes with the aryl hydrocarbon receptor nuclear translocator (Arnt) protein, and binds to dioxin response elements (DREs) in the promoter regions of target genes. The liver possesses the highest concentrations of xenobiotic-metabolizing CYPs (Ding and Kaminsky, 2003). Mechanisms for tissue-specific expression are not fully understood. In the liver, the concerted action of different liver-enriched transcription factors plays a role (Schrem et al., 2004). Recently, signaling through the Wnt/β-catenin/T-cell factor (TCF) pathway has been established as a new regulator of hepatic CYP expression (Loeppen et al., 2005; Sekine et al., 2006; Tan et al., 2006). For a review of the pathway, see Willert and Nusse (1998). In the absence of agonistic Wnt molecules, cytosolic β-catenin is bound to a multiprotein complex, where it is phosphorylated by glycogen synthase kinase 3β (GSK3β) and subsequently degraded in the proteasome. Activation of Frizzled receptors by Wnts destabilizes the phosphorylation complex and allows β-catenin to translocate to the nucleus, where it binds to TCF transcription factors, thus facilitating target gene transcription. Deregulation of the β-catenin pathway, mostly by activating mutations of the Ctnnb1 gene (encoding β-catenin) affecting the phosphorylation sites in exon 3, is frequently observed in human and rodent tumors of the liver and other organs (Behrens and Lustig, 2004).

Mouse hepatomas with activated β-catenin express high levels of different CYPs, including AhR-regulated Cyp1a1/2, even in the absence of dioxin-type inducers (Loeppen et al., 2005; Stahl et al., 2005). Mice with hepatocyte-specific knockout of Ctnnb1 display deregulated CYP expression (Braeuning et al., 2009; Sekine et al., 2006; Tan et al.,...
AhR- and β-catenin–dependent signaling interfere in at least two different ways: First, the AhR is a transcriptional target of β-catenin/TCF signaling (Chesire et al., 2004; Hailfinger et al., 2006); second, in the mouse intestine, the ligand-activated AhR acts as an ubiquitin ligase for β-catenin thus priming the protein for degradation (Kawaijiri et al., 2009).

AhR-dependent CYP isofoms, this study was aimed to analyze the interplay of both signaling pathways in the induction of CYP expression in mouse liver.

**MATERIALS AND METHODS**

**Animals.** Mice with albumin promoter-driven hepatocyte-specific Ctbnl knockout were generated as recently described (Giera et al., 2010). Eight-weeks-old males were injected with 50 mg/kg body weight of the AhR agonist 3-methylcholanthrene (3-MC; Supelco, Bellefonte, PA) dissolved in corn oil, at two consecutive days (control: corn oil). Mice were sacrificed 24 h after the second injection. Animals received humane care, and protocols were complied with institutional guidelines. Livers from Tg(lox(pA)βCat)33Y mice with hepatocyte-specific expression of activated β-catenin33Y were available from recent experiments (Braeuning and Schwarz, 2010a,b; Giera et al., 2010).

The Tg(lox(pA)βCat)33Y mouse was generated as follows: The plasmid pβCat33Y/Cneo (Loeppen et al., 2005) was used to amplify the coding sequence of human S33Y–mutated β-catenin using the primers 5′-ACCGGTACCATACTCTTTGTTATGGGATTGC-3′ (forward) and 5′-ACTCTTCAAGAGATGATTTACAGGTCAGTAT-3′ (reverse) and transferred into the expression vector pCALNL5 (RDB No. 1862; RIKEN, Japan) by the use of the GATGATTTACAGGTCAGTAT-3′ cloning accuracy was verified by dideoxy sequencing. BamHI/Pvull-linearized pβCat33Y/CALT5 was injected into fertilized mouse eggs of the B6D2F1 hybrid to generate Tg(lox(pA)βCat33Y) mice, which were identified by PCR-based genotyping. Transgene screening was in pCALNL5 (Buchmann, 2009). Expression was analyzed using a LightCycler instrument (Roche, Mannheim, Germany), and primers are listed in Supplemental Experimental Procedures. 

**Quantitative reverse transcriptase-PCR.** Total RNA was isolated and reverse transcribed by standard methods as recently described (Braeuning and Buchmann, 2009). Expression was analyzed by using a LightCycler instrument (Roche, Mannheim, Germany), and primers are listed in Supplemental Experimental Procedures. Target gene expression was normalized to 18srRNA, Gapdh, and 3-phosphate dehydrogenase were used in combination with appropriate alkaline phosphatase–conjugated secondary antibodies with 25 strokes in a Dounce homogenizer.

**In silico promoter analysis.** Sequences extracted from the NCBI Web site (http://www.ncbi.nlm.nih.gov) were screened for putative transcription factor binding sites using MathInspector software (Genomatix, Munich, Germany). Seven putative DREs were identified in the 5′– regulatory sequence of mCyp1a1 (termed A–G; Fig. 3E). pT81lac/hCYP1A1-5′ wt contained one putative TCF/β-catenin–binding site (T; Fig. 5A).

**Transfections and luciferase assay.** The 55.1c cells were seeded at 25,000 cells/cm² 24 h prior to transfection with luciferase reporter constructs, in combination with pRL-CMV (Promega, Madison, WI) providing constitutive CMV promoter–driven Renilla luciferase expression, using Lipofectamine 2000 (Invitrogen). In some experiments, expression vectors encoding activated β-catenin33Y (pBcat33Y/Cneo) or wild-type AhR (Loeppen et al., 2005) were cotransfected (control: transfected with the respective empty vector). β-Catenin activity was monitored via the 8× TCF/β-catenin–driven luciferase reporter SupertopFlash (STF). Luciferase activities were measured 48 h after transfection using the Dual Luciferase Kit (Promega). Firefly luciferase activity values were normalized to Renilla luciferase.

**Immunohistochemistry.** Ten micrometers of frozen liver sections were incubated with antibodies against CYP1A1, CYP2C, CYP2E1, or glutamine synthetase (GS) in combination with a horseradish peroxidase–conjugated secondary antibody with 3-amino-9-ethylcarbazole/H2O2 as substrates. Antibodies are listed in Supplemental Experimental Procedures.

**Immunoprecipitation.** Whole-cell homogenates (2 mg protein/500 μl) were incubated with an anti-AhR antibody (2 μg/ml; Biomol, Hamburg, Germany) overnight at 4°C. Antibody–antigen complexes were precipitated using protein Gagarose beads (Roche). Free β-catenin was detected in lysates (20 μg protein/reaction) from LiCl- or NaCl-treated 5L cells by fishing with GST-ECT (cytoplasmic tail of E-cadherin) or GST-TFC4 (N-terminal domain of TFC4) fusion proteins as recently described (Luckert et al., forthcoming).

**Chromatin immunoprecipitation.** The 55.1c cells were transfected with 15mM LiCl for 24 h and/or 1 mM TCD2 for 2 h. Fixing with 1% formaldehyde for 30 min was stopped with 125mM glycine. Cells were harvested and lysed in protease inhibitor–supplemented swelling buffer with 25 strokes in a Dounce homogenizer.
homogenizer. Nuclei were pelleted and resuspended in sonication buffer. DNA was sheared by sonication to 200–800 bp fragments as measured by gel electrophoresis. Insoluble material was pelleted and diluted 1:10 in chromatin immunoprecipitation (ChIP) buffer. An aliquot was set aside as input sample, and the lysate was then precleared for 1 h with Protein-A/G sepharose (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-AhR antibody (4 μg; Santa Cruz Biotechnology [note: a precipitation of minor portions of other proteins cannot be excluded with absolute certainty because under the denaturing conditions of Western analysis, the antibody exhibits some unspecific binding. However, the fact that a strong increase in signal intensity was observed at the DREs of the Cyp1a1 promoter after treatment with the AhR agonist TCDD [Fig. 3] demonstrates that specific signals were detected in the ChIP experiments]) was added to the sample and incubated overnight at 4°C. Immunocomplexes were isolated by adding protein-A/G Sepharose blocked with sonicated salmon sperm DNA and bovine serum albumin and washed 2 × each in washing buffers A, B, C, and TE buffer for 10 min each. Bead-bound complexes were eluted by 10-min incubation at 65°C in elution buffer. NaCl was added to a final concentration of 200 mM, and cross-links were reversed by incubation at 65°C for 5 h. Protein and RNA were digested enzymatically by Proteinase K and RNase A (10 μg each) for 1 h at 37°C. DNA was then isolated by phenol/chloroform/isoamylalcohol extraction and ethanol precipitation. The aliquot reserved as input DNA was treated exactly as the immunocomplexes were after washing. Real-time PCR was undertaken using either input DNA or immunoprecipitated DNA (± antibody), and primers (P1–P4; see Supplemental Experimental Procedures) were designed to amplify regions of the mCyp1a1 promoter (Fig. 3E). As a control, PCR was also performed using primers (P5) that recognize downstream sequences of the coding region of Cyp1a1 to demonstrate specificity of the pull-down. The amount of the respective Cyp1a1 promoter DNA (P1–P4) in the samples was normalized to the values obtained with P5 primers. Composition of all buffers is contained in Supplemental Experimental procedures.

Statistical analysis. For statistical analysis, Student’s t-test was used. Differences were considered significant when p < 0.05.

RESULTS

β-Catenin Activation Increases CYP Expression

Primary mouse hepatocytes were treated with medium enriched in Wnt3A, a physiological activator of β-catenin signaling. Wnt3A strongly induced the known Wnt/β-catenin target messenger RNAs (mRNAs) Axin2 (−95-fold) and Gpr49 (−35-fold) (data not shown) and caused a statistically significant upregulation of all six CYP mRNAs analyzed (Fig. 1A). This was accompanied by elevated mRNA levels of the nuclear receptors AhR and pregnane X-receptor, whereas no significant effect on constitutive androstane receptor mRNA was detected (Fig. 1B). However, all three receptors were significantly upregulated in mouse hepatomas with activated β-catenin (data not shown). Comparable upregulation of CYPs and the AhR was observed in hepatocytes following inhibition of GSK3β (data not shown).

In vitro stimulation of CYP expression by β-catenin is in line with in vivo observations in transgenic mice with hepatocyte-specific expression of mutant activated β-cateninS33Y. In these mice, β-catenin activation is indirectly suggested to have occurred in a number of hepatocytes distributed in a scattered manner within the liver lobule by the immunohistochemical demonstration of the coexpression of several known β-catenin target genes, which normally would, because of the midzonal and periportal localization of these cells within the liver lobule, not be expressed (Braeuning and Schwarz, 2010a,b; Giera et al., 2010; own unpublished observations). Among these genes are GS and several CYP isoforms: Transgenic
hepatocytes show ectopic expression of GS, CYP1A, CYP2C, and CYP2E1 (Fig. 1C). Interestingly, analysis of optical density revealed higher intensity of CYP1A immunoreactivity in transgenic hepatocytes than in normal appearing pariventricular hepatocytes with physiological CYP1A expression (Fig. 1D). By contrast, mice with hepatocyte-specific Ctnnb1 knockout displayed reduced expression of different CYP mRNAs/proteins and possessed less AhR mRNA than wild-type mice (Figs. 1E and 1F). AhR protein levels, however, exhibited remarkable interindividual variance but did not differ between genotypes (Fig. 1F).

The well-characterized Cyp1a1/2-AhR system was chosen to further analyze the interplay of β-catenin and CYP regulation. In vitro studies were focused on Cyp1a1 because, for unknown reasons, expression and inducibility of Cyp1a2 is poorly preserved in mouse hepatocyte cultures.

### β-Catenin Augments Cyp1a1 Induction by Ligand-Activated AhR

Having shown that β-catenin signaling controls basal Cyp1a1 expression (i.e., in the absence of AhR-activating compounds), we next analyzed whether β-catenin would also affect xenobiotic-induced expression enforced by ligand-activated AhR. Primary hepatocytes were pretreated with Wnt3A and then exposed to the potent AhR activator TCDD. Priming with Wnt3A significantly augmented transcriptional activation of Cyp1a1 by TCDD (Fig. 2A). This is complemented by in vivo data from mice with hepatocyte-specific Ctnnb1 knockout, where Cyp1a1/2 mRNA induction by the AhR agonist 3-MC was alleviated (Fig. 2B).

Mouse hepatoma cell lines 55.1c and Hepa1c1c7 were chosen to further analyze the role of β-catenin signaling for activation of AhR-mediated transcription. A heterozygous deletion within exon 3 of Ctnnb1 leads to constitutive but non-maximum activation of β-catenin signaling in 55.1c, as can be taken from the fact that transfection of a plasmid encoding activated β-cateninS33Y significantly induces the activity of the β-catenin–dependent luciferase reporter STF (see below). The 55.1c cells had been used in previous studies to analyze β-catenin–dependent effects on the rCyp2b1 (Loeppen et al., 2005) and mGSTm3 promoters (Giera et al., 2010). Hepa1c1c7 cells solely express exon 3–deleted β-catenin, leading to constitutive maximal activity of the pathway (Braeuning and Buchmann, 2009). After transfection with anti-Ctnnb1 siRNA, a knockdown of Ctnnb1 by −50% (55.1c) and −80% (Hepa1c1c7) was achieved, corresponding to −50% downregulation of Axin2 expression and STF reporter activity (data not shown; see also data in Braeuning and Schwarz, 2010b). Under these conditions, basal expression of Cyp1a1 as well as the extent of induction after TCDD treatment was significantly alleviated in both cell lines when compared with cells transfected with scrambled control siRNA (Figs. 2C and 2D). AhR mRNA levels were concomitantly lowered by Ctnnb1-specific siRNA in Hepa1c1c7 but not in 55.1c (Figs. 2C and 2D). Inversely, the effects of β-catenin activation on AhR expression were also analyzed in 70.4 mouse hepatoma cells, which are wild-type with respect to Ctnnb1. On average, stabilization of endogenous wild-type β-catenin by 15mM of the GSK3β inhibitor LiCl for 24 h resulted in a −2.5-fold upregulation of Axin2 mRNA expression, which was accompanied by a −1.65-fold upregulation of AhR mRNA (data not shown).

### β-Catenin Signaling Enhances AhR-Dependent Transcription Without Increasing AhR-DNA Interaction

The interplay of β-catenin– and AhR-dependent transcription was analyzed using artificial reporter systems containing either 3X AhR/Arnt-responsive DRE motifs (DRE reporter) or 8X TCF/β-catenin–binding sites (STF). An expression vector encoding activated β-cateninS33Y was cotransfected. Expression of β-cateninS33Y, but not TCDD treatment, stimulated the STF reporter by approximately threefold (Fig. 3A). Inversely, TCDD strongly stimulated the DRE reporter, whereas only a slight statistically nonsignificant stimulation was observed by cotransfection of the β-cateninS33Y expression vector (Fig. 3B). Accordingly, basal activity of the DRE reporter was also not influenced by LiCl treatment of 55.1c, Hepa1c1c7, and 5L cells (data not shown; see also data in Braeuning and Buchmann, 2009). Interestingly, expression of β-cateninS33Y doubled the inducing effect of TCDD on the DRE reporter (Fig. 3B). Transfection of an AhR expression vector did not mimic the costimulatory β-cateninS33Y effect on TCDD-mediated DRE activity (data not shown). When β-catenin signaling was knocked down by siRNA in TCDD-stimulated 55.1c cells, the DRE reporter signal declined significantly (Fig. 3C), resembling the findings from the mRNA analyses (Fig. 2). The rather low basal DRE reporter signal was not significantly influenced by transfection of the Ctnnb1-specific siRNA (Fig. 3C).

Superstimulation of the DRE reporter after simultaneous activation of β-catenin and the AhR might be caused by increased amounts of DNA-bound AhR or by elevated transactivation potential of unaltered amounts of DNA-bound receptor. Therefore, ChIP analysis of AhR binding to DREs in the murine Cyp1a1 promoter was performed. In silico analysis revealed several putative DREs (termed A–G) in the proximal 1500 bp of the promoter, as schematically shown in Figure 3E. PCR amplicons used for analysis of precipitated DNA are also indicated. For ChIP analyses, 55.1c cells were treated with 15mM of the GSK3β inhibitor LiCl for 24 h to activate β-catenin signaling (Fig. 3D) and/or with 1nM TCDD for 2 h to activate the AhR. LiCl treatment was accompanied by an −1.6-fold induction of Cyp1a1 mRNA levels (data not shown). It has to be noted in this context that LiCl does not induce Cyp1a1 in primary hepatocytes from mice with liver-specific
knockout of Ctnnb1 as it also does not in Hepa1c1c7 cells, which exclusively express constitutively activated β-catenin (Braeuning and Buchmann, 2009). Thus, even if LiCl may exert certain effects independent of GSK3β and β-catenin (Pasquali et al., 2010), the regulation of Cyp1a1 is very likely to be mediated by these two proteins. TCDD led to a specific increase in DNA binding of the AhR at the DRE cluster B–E (primers P2–P3) and, to a lesser extent, at DREs A and F (P1 and P4) (Fig. 3F). β-Catenin activation alone did not meaningfully alter DNA binding of the AhR. Moreover, AhR-DNA interaction was not boosted in cells treated with LiCl plus TCDD, indicating that β-catenin signaling does not interfere with AhR-DNA binding (Fig. 3F).

Physical Interaction of AhR and β-Catenin

These results pointed toward an increased transactivation potential of the AhR after β-catenin activation. A physical interaction between the AhR and β-catenin, acting as transcriptional coactivator, seemed possible and was analyzed by immunoprecipitation using the 5L rat hepatoma cell system. This system constitutes of AhR-wild-type 5L, thereof-derived AhR-deficient BP8, and stably AhR-retransfected BP8+ cells, providing the opportunity to perform analyses in the presence or absence of the AhR. Moreover, AhR protein levels are much higher in 5L cells as compared with our mouse hepatoma lines (data not shown). LiCl was used at a concentration of 15mM to induce β-catenin signaling because 5L and thereof-derived cells were refractory to Wnt3A. LiCl significantly induced the expression of the model β-catenin target geneAxin2 by ~1.5-fold and induced Cyp1a1 mRNA in 5L and BP8+ cells by approximately five to sevenfold but not in BP8 cells (Fig. 4). As mentioned above, activity from the DRE reporter was not affected by LiCl in 5L cells (data not shown).

FIG. 2. Influence of Wnt/β-catenin signaling on AhR-mediated Cyp1a1 induction. (A) Induction of Cyp1a1 mRNA by the AhR agonist TCDD in cultured mouse hepatocytes after priming with Wnt3A. Mean ± SD are depicted (n = 5). (B) Cyp1a1/2 expression in livers of Ctnnb1 wild-type (wt) and knockout (ko) mice in response to the AhR agonist 3-MC. Mean ± SD are depicted (n = 5). Influence of siRNA directed against Ctnnb1 on basal and TCDD-induced Cyp1a1 and AhR expression in 55.1c (C) and Hepa1c1c7 cells (D). Mean ± SD are given (n = 9). Control, unspecific scrambled siRNA; ctnnb1, β-catenin–specific siRNA.
FIG. 3. Effects of β-catenin activation on AhR/DRE-dependent transcription in 55.1c cells. (A) Activity of the 8× β-catenin/TCF-driven STF luciferase reporter after cotransfection of a plasmid encoding activated β-cateninS33Y (S33Y) and after TCDD treatment; n.d., not determined. (B) Effect of β-cateninS33Y and TCDD on a 3× AhR/DRE motifs–driven luciferase reporter. (C) Influence of siRNA directed against Ctnnb1 on basal and TCDD-induced DRE reporter activity. Control, unspecific scrambled siRNA; ctnnb1, β-Catenin–specific siRNA. For luciferase experiments, mean ± SD are given (n = 3–4; each performed in quadruplicate). (D) Accumulation of the wild-type β-catenin protein after treatment with 15mM LiCl demonstrates the efficacy of GSK3β inhibition/β-catenin activation in 55.1c cells; wt, wild type protein; del, exon 3–deleted protein. (E) Schematic representation of a 1.5-kb fragment of the murine Cyp1a1 promoter. Putative DRE sites and PCR amplicons (P1–P5) used in the ChIP assay are indicated. (F) Recruitment of AhR to the Cyp1a1 regulatory region, as determined by ChIP analysis. Cells were treated with the β-catenin activator LiCl and/or TCDD. AhR-DNA complexes were precipitated using anti-AhR antibodies (Ab). Quantitative real-time PCR of DNA from the immunoprecipitated fractions was performed using primers that amplify the promoter region as well as the coding region of mCyp1a1 as indicated. Enrichment of promoter DNA was normalized to that of the coding sequence in each sample. Mean ± SEM are given (n = 4).
Extracts from LiCl-treated and control cells were precipitated using an anti-AhR antibody and analyzed by Western blotting, using anti-β-catenin and anti-AhR antibodies. Representative results are shown in Figure 4A: Low amounts of β-catenin were detectable in all samples, including those from AhR-deficient BP8, due to unspecific binding of the protein to the agarose beads. Activation of β-catenin signaling by LiCl triggered a pronounced increase in the amount of β-catenin coprecipitated by the AhR-specific antibody in samples from cell lines 5L and BP8+. This was not observed in samples from AhR-deficient BP8 cells. This suggested that a specific physical interaction between the two proteins had occurred in 5L and BP8+ cells in response to treatment with LiCl. The lack of increased binding of β-catenin to the beads in LiCl-treated BP8 demonstrates that the observed effect is not caused by enhanced levels of free β-catenin binding unspecifically to the agarose beads. Total cellular β-catenin protein levels in response to LiCl were unchanged (Figs. 4B and 4C), whereas levels of free β-catenin increased upon LiCl treatment, as determined by GST-ECT and GST-TCF4 fishing. This was accompanied by an increase in nonphosphorylated β-catenin, a decrease in phospho-S33/S37/T41 β-catenin (Figs. 4D and 4E), and an elevated Axin2 expression (Fig. 4F).

A β-Catenin/TCF DNA-Binding Motif Is Involved in hCYP1A1 Promoter Stimulation by β-Catenin

The involvement of distinct transcription factor binding sites in β-catenin-dependent regulation of basal and inducible CYP1A1 transcriptional rate was analyzed using a luciferase reporter driven by a 1.2-kb fragment of the 5′-upstream sequence of the human CYP1A1 promoter (Schreiber et al., 2006). This region contains the four functional DREs, termed C, D, E, and F (Kress et al., 1998), and a single putative β-catenin/TCF site identified in silico. Different variants of the CYP1A1 reporter plasmid came into operation: the wild-type construct (wt), a mutant version (named CDEF) lacking the DREs, a second mutant (T) lacking the TCF site, and a variant (CDEFT) with all five binding sites knocked out by site-directed mutagenesis; for illustration, see Figure 5A.

After transfection into 55.1c cells, the wild-type hCYP1A1 promoter construct was slightly but significantly (~1.75-fold) induced by cotransfection of activated β-catenin533Y (Fig. 5B). Comparable activation of the reporter was observed when cells were cotransfected with the CDEF mutant and the β-catenin533Y expression vector. By contrast, β-catenin533Y-mediated induction of luciferase activity was largely lost when testing the reporter plasmids lacking the TCF-binding site (T, CDEFT). In addition, activity of hCYP1A1-derived reporters was monitored in response to AhR activation by TCDD. As expected, activity of the wild-type reporter was markedly stimulated by TCDD (Fig. 5C). This effect was clearly dependent on the presence of functional DREs because their mutational inactivation in the CDEF mutant completely abolished responsiveness to TCDD. Interestingly, luciferase reporter activity was stimulated up to significantly

FIG. 4. Physical AhR-β-catenin interaction in 5L rat hepatoma cells and thereof-derived mutants BP8 (AhR-deficient) and BP8+ (stably AhR-transfected BP8). Cells were incubated with the β-catenin activator LiCl and AhR-β-catenin interaction was analyzed by immunoprecipitation. (A) Western analysis of β-catenin (β-Cat) and AhR protein contents in anti-AhR antibody–precipitated (AhR-AB) protein fractions. (B) Overall β-catenin (β-Cat) protein levels in 5L and cognate cell lines after LiCl treatment. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (C) Overall β-catenin protein levels in LiCl-treated 5L cells, as determined by sandwich immunoassay. LiCl stimulated β-catenin signaling in 5L cells, as indicated by the increase in nonphosphorylated and the decrease in S33/S37/S41-phosphorylated β-catenin (D), by the increased interaction with the cytoplasmic domain of E-cadherin (GST-ECT fishing assay) and TCF4 (GST-TCF4 fishing assay) (E), and by the induction of the β-catenin target gene Axin2 (F). (G) Transcriptional response of 5L, BP8+, and BP8 rat hepatoma cells to stimulation of β-catenin signaling by LiCl (15mM, 24 h) and stimulation of the AhR by TCDD (1nM, 24 h). LiCl stimulated Cyp1a1 mRNA expression in AhR-proficient 5L and BP8+ cells but not in AhR-deficient BP8. (H) AhR mRNA levels, which were overall higher in 5L than in BP8+, were not affected by LiCl. Mean ± SD are depicted (% n = 3); n.d., not detectable.
DISCUSSION

The present data demonstrate that β-catenin (1) induces basal transcription of various CYP isoforms, including AhR-regulated Cyp1a1/2; (2) has a striking costimulatory effect on TCDD-mediated Cyp1a1 induction; and (3) increases the activity of the hCYP1A1 promoter in a TCF site–dependent but DRE-independent manner. These results point toward a complex network of β-catenin/AhR interactions as schematically delineated in Figure 6.

FIG. 5. Effects of β-catenin activation and TCDD treatment on human CYP1A1 promoter–driven luciferase reporter activity in 55.1c cells. (A) Schematic representation of the 1.2-kb promoter construct containing four functional DREs (CDEF) and a putative TCF/β-catenin–binding site (T). (B) Cells were transfected with wild-type (wt) and point-mutated (CDEF, mutations of all functional AhR-responsive elements [DRE]; T, mutation of the TCF/β-catenin site; CDEFT, mutation of the DREs and the TCF/β-catenin site) promoter luciferase reporters in combination with a plasmid encoding activated T-plakinS33Y (S33Y). (C) Effects of promoter mutations on TCDD-inducible luciferase reporter activity. For all experiments, mean ± SD are given (n = 4; each performed in quadruplicate).

higher levels by TCDD in the native construct as compared with the T plasmid lacking the TCF/β-catenin site (Fig. 5C).
FIG. 6. Schematic delineation of β-catenin signaling and (partially putative) AhR/β-catenin interactions. Physiologically, β-catenin (Cat) is connected to membrane-bound E-cadherin (Ecad). Free cytosolic β-catenin is phosphorylated by a multiprotein complex containing, among others, GSK3β (GSK) and the adenomatous polyposis coli protein (Apc) and is subsequently degraded. Binding of Wnts to Frizzled (Fzd) receptors inhibits β-catenin phosphorylation, thus enabling its nuclear translocation. In the nucleus, β-catenin associates with TCF transcription factors activating target genes, such as AhR and Cyp1a1. Upon activation by a ligand (T), the AhR binds to DREs together with its partner Arnt. β-Catenin cooperatively facilitates AhR/Ant/ DRE-dependent transcription by its activity at TCF sites and by enhancing transactivation potential of DNA-bound AhR, possibly by direct interaction.

signaling by ligand-activated AhR in mouse liver. This suggests highly tissue-specific mechanisms regulating AhR/β-catenin interactions.

Another level of β-catenin/AhR interplay may occur via interactions of DREs and TCF sites. We have shown that several known DREs in the hCYP1A1 promoter, which are required for TCDD-induced activation of hCYP1A1, are dispensable for β-catenin–mediated activation of the reporter. By contrast, a newly identified TCF site is involved in reporter activation by both, β-catenin and TCDD/AhR, at least under the in vitro conditions used in our analyses (Fig. 5). However, β-catenin activity alone was not sufficient for Cyp1a1 induction from the native promoter in the AhR-deficient BP8 cells (Fig. 4), suggesting that some background AhR activity (putatively driven by yet unidentified endogenous ligands) might be needed to facilitate access of β-catenin/TCF to the Cyp1a1 promoter. On the other hand, it appears possible that local activity of β-catenin/TCF at the TCF site facilitates accessibility of neighboring DREs for ligand-activated AhR/Arnt, which would explain the enhancing effect of β-catenin on TCDD-induced gene transcription. In consequence, this will lead to a model where strong β-catenin activity (supported by low background AhR signaling) determines basal Cyp1a1/2 expression while contemporaneously increasing the susceptibility to exogenous AhR agonists such as TCDD.

CYPs and other drug-metabolizing enzymes are zonally expressed within the liver lobule, and the highest expression is generally found in the perivenous hepatocyte subpopulation (Lindros, 1997). Perivenous hepatocytes also possess activated β-catenin (Benhamouche et al., 2006; Sekine et al., 2007) and are the preferential target for enzyme-inducing xenobiotics such as TCDD and other exogenous compounds (Anderson et al., 1989). Our observation that β-catenin facilitates DRE-dependent gene expression, either by direct β-catenin/AhR protein interactions or by interference at the level of DNA-binding sites, may therefore offer a mechanistic explanation for the increased susceptibility of perivenous hepatocytes to AhR-activating xenobiotics.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci. oxfordjournals.org/.

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