A β-catenin-dependent pathway regulates expression of cytochrome P450 isoforms in mouse liver tumors

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Phenobarbital (PB) is a model tumor promoter in the rodent liver. In the mouse, the promotional effect of PB results from a selective stimulation of clonal outgrowth of hepatocytes harboring activating mutations in the β-catenin (catnb) gene. Glutamine synthetase (GS), a downstream target in the Wnt/β-catenin/T-cell factor (TCF) signaling pathway, is strongly up-regulated in catnb-mutated mouse liver tumors and may serve as a marker for their identification. Here we show that the levels of several cytochrome P450 (CYP) isoenzymes are also altered in GS-positive liver tumors. Immunohistochemical and western blotting analyses demonstrated that GS-positive, catnb-mutated tumors showed levels of CYP1A, CYP2B, CYP2C and CYP2E1, which were similar or slightly enhanced in comparison with non-tumoral liver tissue. This contrasts with tumors without catnb mutations, which exhibited decreased levels of these CYP isoforms. Real-time RT-PCR revealed that the differences in CYP levels in the tumors corresponded to changes in the respective mRNAs. Mouse hepatoma cells were transiently transfected with an expression vector encoding an S33Y-mutated β-catenin protein, which was functional with regard to transactivation of a β-catenin/TCF-responsive (topflash) reporter construct. Co-transfected with luciferase reporter vectors containing either the regulatory upstream sequence of the CYP2B1 gene or three dioxin-responsive core elements were activated by S33Y-β-catenin. These results indicate that mutation of catnb leads to transcriptional activation of CYP isoenzymes in mouse liver tumors. As CYPs are involved in both the activation and the inactivation of several clinically important anticancer drugs, our findings may be relevant for chemotherapy of human cancers, where activation of β-catenin-dependent signaling by mutation of the gene or alternative mechanisms is frequently observed.

Introduction

The cytochrome P450 (CYP) superfamily consists of a group of enzymes that play a major role in the metabolism of numerous chemicals, including drugs, carcinogens, steroids and natural products. CYPs are expressed in a tissue-specific manner and many of them can be induced by xenobiotics using transcriptional or post-transcriptional mechanisms, whereas others are constitutively expressed. Transcriptional activation is mediated by several nuclear receptors that act as ligand-dependent transcription factors (1-4). The aryl hydrocarbon receptor (AhR) is responsible for induction of CYP1A1 family members by polycyclic aromatic hydrocarbons. Without a ligand, the receptor protein resides in the cytosol in an inactive complex with the heat shock protein Hsp90. Upon ligand binding the receptor translocates to the nucleus and binds to xenobiotic-responsive elements (XRE; also called DRE, dioxin responsive core elements) in the regulatory region of target genes as a heterodimer with another protein called Arnt. XREs are located in the regulatory upstream sequence of several genes including CYP1A1 and several phase II enzymes (GST, NQO1, UGT1A6). Induction by phenobarbital (PB) is mediated by the constitutive active (or androstane) receptor (CAR). Activation and nuclear translocation of CAR by PB is not fully understood, but includes phosphorylation/dephosphorylation events. In the nucleus, CAR heterodimerizes with the retinoid receptor (RXR) and binds to PB-responsive enhancer modules in the upstream region of CYP2B genes.

Pre-neoplastic liver foci, liver nodules and hepatocellular tumors often display aberrant CYP expression. Many CYP isoenzymes are down-regulated in chemically induced liver lesions when compared with the surrounding normal tissue (5-9), and several CYP isoenzymes are also negatively regulated in human hepatocellular cancers (10). Certain CYP isoforms, however, such as CYP2A5, are over-expressed in mouse liver lesions (11,12) and elevated expression of CYP1B1 has also been demonstrated in a variety of human tumors (13-15). Moreover, certain CYP isoforms have been shown to be inducible by typical CYP-inducers such as PB or 3-methylcholanthrene (3-MC) in rodent liver tumors (7,8,16,17).

The molecular mechanisms responsible for tumor-specific alterations in CYP expression are mainly unknown but may be linked to aberrations in genes that are of central importance for the regulation of cellular transcription, proliferation and differentiation. Among these genes are the protooncogenes ras and β-catenin, which are activated by point mutation or partial truncation in many tumors, including mouse liver tumors. Transfection of oncogenic Ha-ras into hepatoma cells has been shown to result in down-regulation of CYP1A1 demonstrating that activation of the ras-dependent pathways can interfere with the expression of this enzyme (18). In contrast, β-catenin has not yet been linked, to our knowledge, to changes in expression of drug metabolizing enzymes. The β-catenin gene catnb encodes a highly conserved protein, which exerts dual roles in cells (for recent reviews see refs 19-23). At the cell membrane, β-catenin is associated with the intracellular domain of E-cadherin, a component of the
adherens junction, and the actin cytoskeleton, and thus plays an important role in cell–cell adhesion. In addition, cytoplasmic β-catenin is a critical downstream target of Wnt signaling. β-Catenin levels in cells are negatively regulated by a multi-protein complex that contains several proteins including glycogen synthase kinase 3β (GSK3β), axin and the adenomatous polyposis coli (APC) protein. Phosphorylation of certain clustered serine/threonine residues at the N-terminal region of β-catenin by GSK3β targets the protein for ubiquitin/proteasome-dependent degradation. Wnt signaling disassembles the destruction complex and prevents β-catenin from phosphorylation, ubiquitination and, hence, degradation. Accumulated β-catenin then enters the nucleus and mediates transcriptional responses by interacting with HMG box transcription factors of the T-cell factor/lymphocyte enhancer–binding factor (TCF/LEF) family. Over 60 target genes are transcription factors of the T-cell factor/lymphocyte enhancer-promoter region linked to the Gκ promoter (22, 23). It has also been shown that only adenomas and highly differentiated carcinomas with an eosinophilic phenotype show nuclear accumulation of β-catenin (33). These results suggested that nuclear translocation of β-catenin and activation of Wingless/Wnt signaling may provide a growth advantage for hepatic tumors with a more differentiated phenotype. In an experimental system including PB as a tumor promoter, activation of β-catenin conferred proliferative and invasive advantages to liver tumors by enhanced expression of matrix metalloproteinase 7 and transmembrane mucin 1 (34).

In a previous study, we have shown that PB, a model tumor promoter that enhances hepatocarcinogenesis in rodents, selectively stimulates the clonal growth of catnb-mutated mouse liver tumors that were initiated by a single treatment with the liver carcinogen N-nitosodimethyamine (DEN). Catnb mutations were present in ~80% of liver tumors from animals promoted with PB, whereas the tumors from mice treated with the initiating carcinogen alone were few. Furthermore, glutamine synthetase (GS), which has recently been shown to be a transcriptional target of β-catenin (36), was strongly up-regulated in tumors harboring catnb mutations, whereas tumors without catnb mutations were negative for GS expression (37).

In the present study, we show that GS-positive liver tumors from mice also exhibit enhanced expression of several CYP450 isofoms, which is due to a direct or indirect transcriptional activation of CYP genes by β-catenin.

Materials and methods

Tissue samples

Mouse liver samples were obtained from two previous experiments with connexin32 (Cx32) wild-type and knockout mice (C57BL/129Sv × C3H/He), which were aimed to investigate the role of Cx32 in tumor promotion by PB (38). Mouse liver samples from animals with the Cx32 wild-type used in the present study. In experiment 1 (promotion study), mice were i.p. injected with a single dose of DEN (90 μg/g body wt) at 6 weeks of age, whereas 10 μg/g body wt of the carcinogen were injected into 12–15-day-old mice in experiment 2 (inhibition study). DEN-treated mice were then kept on a standard diet or on a diet containing 0.05% PB until death, which was 39 weeks (experiment 1) or 25 weeks (experiment 2) after the start of PB treatment. Histologically visible liver tumors (diameters > 3 mm) were collected and frozen in liquid nitrogen; the remaining livers were frozen on blocks of dry ice and stored at ~80°C.

Immunohistochemistry and western analysis

GS was stained immunohistochemically in frozen liver sections (10 μm) by standard protocols (37) using a mouse anti-GS monoclonal antibody (1:2000; BD Biosciences Europe, Heidelberg, Germany), anti-mouse-IgG secondary antibody conjugated with alkaline phosphatase (Sigma-Aldrich, Taufkirchen, Germany) and 3-amino-9-ethylcarbazole/H2O2 as substrates. Various CYP isoforms were stained accordingly using rabbit antiserum against CYP1A, CYP2B, CYP2C (1:300 each; gift of Dr R. Wolf, Biomedical Research Centre, University of Dundee, Dundee, UK; for specificity see ref. 39) and CYP2E1 (1:500; gift of Dr M. Ingelman-Sundberg, Karolinska Institute, Stockholm, Sweden) and an anti-rabbit-IgG secondary antibody.

Western blots with antibodies (1:2000 dilution) against GS, CYP1A, CYP2B, CYP2C, CYP2E1, CYP3A1 (BioTrend Chemikalien GmbH, Cologne, Germany), RXR (Santa Cruz Biotechnology, Santa Cruz, CA), β-catenin (Transduction Laboratories, Lexington, KY; 1:500) and histone deacetylase 2 (HDAC2) (1:500; gift of Dr M. Goetlich, GSF, Neuherberg, Germany) were performed as described recently (35). Antibody binding was visualized using appropriate alkaline phosphatase-conjugated secondary antibodies and CDP-Star as a substrate (Tropix, PE Applied Biosystems, Warrington, UK). Chemiluminescence signals were monitored by use of a CCD camera system and quantified by use of TINA 8.2 V.9.0 software (Raytest, Straubenhardt, Germany).

Cell culture

The mouse hepatoma line 55clc used in the present study was established from a mouse liver tumor (for details on cell line characterization see refs 40, 41). Cells were grown in Dulbecco’s Modified Eagle’s medium (high glucose) containing 10% FCS, 0.02% L-arginin and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin) in a humidified incubator containing 5% CO2 at 37°C as described (40). Cells were routinely passaged twice a week by trypsinization.

Transient transfections and reporter gene assays

The following expression vector were provided for use. The full-length β-catenin harboring an activating S33Y point mutation (pBcatS33Y/Cneo) and the β-catenin/Tcf (top/flash) reporter were provided by Dr O. Müller (Max-Planck Institute of Molecular Physiology, Germany). p5′-CYP2B1-Luc reporter (pGL3C2B1) was provided by Dr K. Hirsch-Ernst (University of Göttingen, Germany); the Ah receptor (pDR/CMV4) by Dr L. Ploelting (Karolinska Institute, Stockholm, Sweden); and mCAR (pcRmCAR-I) was provided by Dr M. Negishi (National Institute of Environmental Health Sciences, NIH, Research Triangle Park, NC). The pT81LUC/DRE reporter was constructed by cloning three copies of a functional DRE motif and adjacent bases (motif DRE3 in ref. 42) in tandem into XhoI and HindIII sites of pT81LUC (43). Cells of line 55clc were plated in 24-well plates at a density of 1 × 105 cells/well (~60% confluency) 24 h before transfection. Cells were transiently transfected with various combinations of plasmid DNAs using 3.5 μl GenePORTER™ reagent (Poqlab, Erlangen, Germany)/well. pRL-CMV (10 ng) containing the CMV immediate-early enhancer/promoter region linked to the Renilla luciferase gene (Promega), was co-transfected for normalization. The amount of DNA was kept constant at 1.1 μg DNA (650 ng of expression plasmids, 440 ng of firefly reporter gene constructs, 10 ng pRL-CMV) per 500 μl medium/well.

Cells were incubated with or without PB (1.5 mM) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (20 μM) for an additional 24 h. In all reporter assays cells were lysed directly on the plate 48 h after transfection. Reporter activities were determined using the dual-luciferase reporter assay system (Promega, Mannheim, Germany). Quantification was performed on a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany). A set of three independent
in tumors from mice of the inhibition study. *Catnb*-mutated lesions can be easily identified by immunohistochemical staining for GS, a transcriptional target of β-catenin (36) that is strongly over-expressed in liver lesions with activated β-catenin but absent in *catnb* wild-type lesions (37). We have now investigated in parallel to GS-stained sections the protein expression levels of several CYP isofoms, namely CYP2B, CYP2C, CYP1A and CYP2E1, by immunohistochemistry using antibodies specific for these families of CYP proteins.

### Immunoohistochemical staining for CYP isofoms

Expression of CYP isofoms is largely down-regulated during the course of chemical hepatocarcinogenesis in rodents, unless animals are treated with enzyme-inducing agents such as PB (5-9). Figure 1A shows a typical example of a CYP2C-immunostained liver section from a mouse that was treated with DEN only. This section contains multiple tumor transections (some marked by arrows), which completely lack CYP2C expression. Other CYP isofoms and the GS marker (see Figure 1A) show the identical negative staining behavior. In PB-treated mice of the promotion study, however, most of the tumors were GS-positive. Figure 1B shows two serial sections through the liver of a PB-treated animal from this study, one stained for GS, the other for CYP2C. Several transections through liver tumors of varying size are clearly identifiable by eye in the GS-stained section. Except one (indicated by a white arrow), all tumors were GS-positive with the majority of cells expressing this enzyme. A corresponding staining pattern was observed for CYP2C. As CYPs of family 2 are inducible by PB, which leads to a strong zonal CYP2C staining of hepatocytes in the surrounding normal liver tissue, over-expression of this enzyme in liver tumors is somewhat less prominent than in the GS-stain. It is remarkable however that the GS-negative tumor also showed reduced CYP2C expression despite the fact that the section was taken from the liver of a PB-treated mouse. Figure 1C shows a representative series of sections through the liver of a second PB-treated mouse of the promotion study. Again, various tumor transections can be easily identified. Previous studies have shown that the GS-positive tumor (b) harbors a single base substitution in codon 35 (T:A→G:C resulting in Iso>Ser exchange) of *catnb*, whereas the GS-negative tumor (a) is wild-type in this gene (37). Clearly, expression of GS and the various CYP isofoms investigated coincided: positive staining for all four CYP isofoms predominated in tumor areas with significant expression of GS, whereas weak or no expression was observed in the GS-negative tumor (a). The PB-inducible CYP2B and CYP2C isofoms show also considerable staining in the normal liver tissue, which is much less pronounced with respect to CYP2E1 and CYP1A, which are not inducible by PB. The levels of both CYP2E1 and CYP1A, however, were clearly elevated in GS-positive tumors. In the rat, neoplastic liver lesions from acutely PB-treated animals exhibit increased levels of PB-inducible but not of 3-MC-inducible CYP isofoms, demonstrating that PB can stimulate the expression of the respective CYP isofoms in the otherwise CYP-negative lesions (7,8). In contrast, GS-positive mouse tumors from PB-treated mice of the present study showed elevated levels of both PB-inducible and 3-MC-inducible CYP isofoms. In the inhibition study, almost all lesions were GS-negative, independent of whether the mice received PB or not (37). Similarly, only very few GS-positive lesions were present in livers from mice of the promotion study not

### Results

The liver tumors used in the present study were derived from two previously performed experiments aimed to investigate the mechanism of tumor promotion by PB in Cx32 wild-type and Cx32 null mice (38). For the present work, only material from the Cx32 wild-type mice was used. In experiment 1 (promotion study), mice were given a single injection of the initiating carcinogen DEN at the age of 6 weeks and were subsequently administered PB via their diet or received the control diet for 39 weeks. Under these conditions, PB significantly promoted hepatocarcinogenesis (38). In contrast, an inhibitory effect of PB on hepatocarcinogenesis was obtained in experiment 2 (inhibition study), where infant instead of adult mice were treated with DEN followed by feeding of a PB-containing or control diet (38). The mechanism of this paradoxical effect of the barbiturate is not entirely clear but similar effects have also been reported by others (46). About 80% of liver tumors from PB-treated mice of the promotion study harbored activating mutations in the *catnb* gene (35). In contrast, the frequency of *catnb*-mutated lesions was very low in tumors from mice not treated with the tumor promoter PB and in tumors from mice of the inhibition study. *Catnb*-mutated lesions can be easily identified by immunohistochemical staining for GS, a transcriptional target of β-catenin (36) that is strongly over-expressed in liver lesions with activated β-catenin but absent in *catnb* wild-type lesions (37). We have now investigated in parallel to GS-stained sections the protein expression levels of several CYP isofoms, namely CYP2B, CYP2C, CYP1A and CYP2E1, by immunohistochemistry using antibodies specific for these families of CYP proteins.
treated with PB. In total, we were able to identify 12, generally rather small, GS-positive lesions from non-PB-treated mice of the promotion study, which all demonstrated a concomitant positive CYP immunostaining. A representative series of sections through such a neoplastic lesion is shown in Figure 1D. We have also observed a concomitantly elevated expression of GS and CYPs in a few GS-positive liver lesions, which could be identified in livers of non-PB-treated mice from additional presently ongoing studies in our laboratory (data not shown).

A quantitative evaluation of expression of CYP2E1, CYP2B, and CYP2C in GS-positive and GS-negative liver lesions from mice of the promotion and inhibition study is presented in Figure 2. Almost all GS-positive tumors isolated from livers of PB-treated animals demonstrated elevated CYP levels and all 12 GS-positive lesions derived from non-PB-treated mice stained positive for CYP2E1 and CYP2C, and nine showed positive CYP2B staining, albeit apparently somewhat weaker than in GS-positive tumors from PB-treated mice. In contrast,

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Fig. 1. Immunohistochemical detection of GS and various CYP isoforms in mouse liver tumors. (A) Serial liver sections from a DEN-treated mouse of the inhibition study stained for GS and CYP2C, respectively. Normal liver tissue shows zonal staining for both enzymes; liver tumors (some indicated by white arrows) entirely lack GS and CYP staining. (B) Serial liver sections from a DEN/PB-treated mouse of the promotion study stained for GS and CYP2C. GS-positive liver tumors express CYP2C; in contrast, GS-negative tumors lack CYP2C staining (white arrow). (C) Series through the liver of a DEN/PB-treated mouse (promotion study) stained for GS and the indicated CYP isoforms. Previous studies (37) have shown that the GS-negative tumor (a) is catnb wild-type whereas tumor (b) harbors a point mutation in the gene. Note the coincidence of positive and negative GS- and CYP-staining in the various liver lesions present in the sections. (D) Serial sections through the liver of a mouse of the inhibition study treated with DEN only; GS and all CYP isoforms demonstrate positive immunostaining in the lesion present in the section; bar: 0.2 mm.
CYP expression was only observed in a minor fraction of the GS-negative tumors: only one out of 114 lesions from non-PB-treated animals was positive for CYP2B, whereas the other two isoforms were negative throughout. Less than 20% of GS-negative lesions from PB-treated mice, which were almost exclusively from the inhibition study, displayed detectable CYP2C and CYP2E1 levels and ~40% were CYP2B-positive. Taken together, the results demonstrate that almost all GS-positive, presumably catnb-mutated lesions show concomitantly elevated CYP expression, whereas only a subpopulation of the lesions shows induction of CYP isoforms independent of activated β-catenin.

**Western analysis**
The levels of GS and CYP isoforms were also quantified by western analysis of homogenates from normal liver and liver tumors. For this analysis, catnb-mutated tumors were only available from PB-treated mice, whereas catnb wild-type tumors were all from non-PB-treated mice of the promotion study. Corresponding normal liver tissues were from PB-treated and non-PB-treated mice. For the analyses, the entire tumor material was used up and therefore not accessible for histological examination. Such analyses, however, were performed in parallel by use of hemalaun/eosin-stained liver sections from PB-treated and untreated mice of the promotion study. In accordance with previous findings (38,46), liver tumors generated in mice in the absence or presence of PB demonstrated considerable differences upon histological examination: the former were often basophilic while the latter were mostly eosinophilic. In accordance with our previous work (37) the levels of the marker GS were strongly increased in catnb-mutated liver tumors but unchanged in catnb wild-type tumors in comparison with normal liver (Figure 3A). PB-treatment of mice did not affect GS levels in normal hepatocytes. Corresponding to the GS marker, the levels of CYP2B, CYP2C and CYP2E1 were much higher in catnb-mutated tumors when compared with...
wild-type tumors whereas only minor differences were seen with respect to CYP3A (Figure 3A). In normal liver, PB increased the expression of CYP2B, as expected, but was either inactive or only weakly active as an inducer on the other CYP isoforms investigated. CYP2C, CYP2E1 and CYP1A1 levels were decreased in catnb wild-type tumors and demonstrated only ~20–40% of the concentrations detected in normal liver tissues from non-PB-treated mice upon a quantification of signals obtained in the western analyses. An exception was CYP3A, which was decreased by only 30%. In contrast, the various CYP isoforms were increased by factors of ~2–3 in catnb-mutated tumors when compared with normal liver tissue from PB-treated mice with the exception of CYP3A, which was almost unchanged.

In accordance with earlier observations (35), the overall concentration of the β-catenin protein was not significantly elevated in catnb-mutated tumors as compared with wild-type tumors. Furthermore, levels of RXR, a protein that has been shown previously to regulate β-catenin levels (47), were not altered by PB treatment in normal liver and were almost identical in all tumor samples. As HDAC2 was found to be induced via Wnt/β-catenin signaling in colonic cancer cells (M.Goettlicher, personal communication), levels of this enzyme were also analyzed. We were not able, however, to detect any differences in expression between samples (Figure 3A).

For analysis of protein levels of the Ah receptor and its target gene(s) CYP1A1/2 we included normal liver samples from a previously performed experiment where mice had been treated with a single injection of 250 ng/kg body wt of TCDD, a potent CYP1A inducer. In normal liver tissue, CYP1A1 levels were low and unaffected by PB treatment, but significantly increased by TCDD. Catnb-mutated liver tumors showed increased CYP1A levels in comparison with normal tissue from non-TCDD-treated mice whereas catnb wild-type tumors demonstrated almost undetectable concentrations of the enzyme (Figure 3B). Interestingly, AhR levels were elevated in both types of tumors in comparison with normal liver (Figure 3B).

### Analysis of mRNA

Quantitative two-step RT–PCR experiments using the Light-cycler instrument were performed to clarify whether up-regulation of GS protein and CYP isoforms results from increased transcription in catnb-mutated tumors. In addition, we studied the effect of PB on the expression of these enzymes in normal hepatocytes. In normal liver from PB-treated mice CYP2B10 mRNA was strongly up-regulated (>300-fold) when compared with tissues from non-PB-treated mice, whereas CYP1A1 mRNA was almost unaffected (1.75-fold increase). The mRNA levels of GS, CYP2E1 and of GAPDH, a housekeeping gene that was included in the analysis for normalization of data, were also not affected by PB. In agreement with data obtained on the protein level, catnb wild-type tumors exhibited decreased CYP mRNA levels in comparison with non-tumoral tissue from mice not exposed to PB, with the exception of the CYP2B10, where this effect was not significant (Table II). Catnb-mutated tumors showed strongly elevated GS mRNA levels (>300-fold), when compared with the normal liver from PB-treated mice. CYP1A1 and CYP2B10 mRNA levels were almost unchanged in these tumors, while CYP2E1 mRNA was increased significantly by a factor of ~2.6 (Table II). Upon comparison of mRNA levels in catnb-mutated tumors with those in their non-catnb-mutated cousins, CYP2B10, CYP1A1 and CYP2E1 were significantly elevated by factors of 300-, 34- and 32-fold (Table II). These results suggested a direct or indirect effect of activated β-catenin on the transcription of GS and certain CYP isoforms in mouse liver tumors.

#### Transient transfection experiments

Induction of CYP1A and CYP2B isoforms by xenobiotics is mediated by AhR and CAR, respectively. The effect of

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**Table II. Relative mRNA quantification of GS and CYP isoforms by real-time PCR**

<table>
<thead>
<tr>
<th>Genes</th>
<th>GS</th>
<th>CYP2B10</th>
<th>CYP2E1</th>
<th>CYP1A1</th>
<th>GAPDH</th>
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<tbody>
<tr>
<td><strong>CP values</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>−PB</td>
<td>1.84</td>
<td>1.67</td>
<td>1.68</td>
<td>1.84</td>
<td>1.87</td>
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<td>Normal liver</td>
<td>25.2 ± 0.8</td>
<td>39.6 ± 3.8</td>
<td>18.0 ± 1.9</td>
<td>33.8 ± 0.6</td>
<td>17.7 ± 1.5</td>
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<tr>
<td>Catnb wild-type tumors</td>
<td>28.2 ± 2.1</td>
<td>42.4 ± 2.5</td>
<td>24.9 ± 4.7</td>
<td>39.9 ± 3.6</td>
<td>19.7 ± 8.2</td>
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<tr>
<td>+PB</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Normal liver</td>
<td>26.2 ± 1.2</td>
<td>27.8 ± 0.2</td>
<td>17.0 ± 0.1</td>
<td>32.5 ± 0.2</td>
<td>17.3 ± 0.2</td>
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<tr>
<td>catnb-mutated tumors</td>
<td>19.2 ± 0.5</td>
<td>31.3 ± 1.8</td>
<td>18.1 ± 2.9</td>
<td>34.1 ± 0.7</td>
<td>19.8 ± 3.5</td>
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Comparison of catnb wild-type tumors with untreated normal liver tissue

<table>
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<tr>
<th>Expression ratios</th>
<th>0.59</th>
<th>0.81</th>
<th>0.10</th>
<th>0.087</th>
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<tr>
<td>P-value</td>
<td>0.604</td>
<td>0.739</td>
<td>0.002</td>
<td>0.045</td>
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Comparison of catnb-mutated tumors with PB-treated normal liver tissue

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<tr>
<th>Expression ratios</th>
<th>331.2</th>
<th>0.77</th>
<th>2.58</th>
<th>1.717</th>
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<tr>
<td>P-value</td>
<td>0.001</td>
<td>0.356</td>
<td>0.016</td>
<td>0.429</td>
<td>−</td>
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Comparison of catnb-mutated tumors with catnb wild-type tumors

<table>
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<tr>
<th>Expression ratios</th>
<th>323.5</th>
<th>301.7</th>
<th>32.7</th>
<th>34.4</th>
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</thead>
<tbody>
<tr>
<td>P-value</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.012</td>
<td>−</td>
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</table>

Comparison of PB-treated to untreated normal liver tissue

<table>
<thead>
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<th>Expression ratios</th>
<th>0.41</th>
<th>320.6</th>
<th>1.28</th>
<th>1.75</th>
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<tr>
<td>P-value</td>
<td>0.051</td>
<td>0.001</td>
<td>0.490</td>
<td>0.047</td>
<td>−</td>
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mRNA levels were determined in two-step real-time RT–PCR analyses. CP values (mean ± SD) of six independent tissue samples are given. Relative expression ratios were calculated with respect to GAPDH as endogenous control, taking into account the respective PCR efficiencies. Statistical analysis was done using a randomization test.
activated β-catenin on transcriptional activation of these proteins was studied in transient transfection assays with CAR- and AhR-responsive luciferase reporter vectors using cells of mouse hepatoma line 55.1c (40). Previous experiments have shown that transfection of 55.1c cells with an expression vector encoding activated β-catenin (S33Y-β-catenin) leads to a strong activation of a β-catenin/TCF-responsive reporter (topflash) (48), an effect, which was also observed in the present study (Figure 4A). Co-transfection of the S33Y-β-catenin expression vector together with a luciferase reporter containing an ~2.7 kb fragment of the 5' flanking promoter region of the rat CYP2B1 gene (pGL3C2B1) (49) resulted in a 13-fold stimulation of reporter activity (Figure 4B). PB, which only moderately stimulated the reporter by itself, was not required to produce this effect but rather caused a slight decrease in S33Y-β-catenin-mediated reporter activation. Co-transfection of a mCAR-1 expression vector (pCR3-mCAR) (50) mediated a slight activation of the pGL3C2B1 reporter, which was not affected by PB. Co-transfection of mCAR-1 together with S33Y-β-catenin did not further enhance S33Y-β-catenin-mediated stimulation of the luciferase reporter and additional incubation with PB had no obvious effect (Figure 4B). In a second series of experiments, a TK-driven luciferase construct containing three DRE-core motifs (pT81LUC3 × DRE) was transfected into 55.1c cells under the same conditions as described above (Figure 4C). This artificial reporter is well suited to quantify AhR-mediated responses in transient transfection assays. TCDD exposure increased the activity of the reporter 20-fold in comparison with untreated control cells transfected with empty expression vector. In contrast to the pGL3C2B1-reporter, however, the DRE-driven reporter was only marginally activated by co-transfected S33Y-β-catenin alone but the activity of the reporter could be stimulated 46-fold in S33Y-β-catenin co-transfected cells that were additionally treated with TCDD (Figure 4C). Co-transfection with an AhR expression vector enhanced

**Fig. 4.** Effect of over-expression of activated β-catenin on the activity of PB- and TCDD-responsive luciferase reporters in mouse hepatoma cells. (A) 55.1c mouse hepatoma cells were transiently transfected with an expression vector coding for S33Y-β-catenin and a β-catenin/TCF-responsive luciferase reporter vector (topflash). (B) 55.1c cells were transiently transfected with expression vectors for S33Y-β-catenin and CAR as indicated, together with a luciferase reporter vector containing ~2.7 kb of the upstream regulatory region of the CYP2B1 gene. Transfected cells were either left untreated or exposed to 1.5 µM PB for 24 h. Results are expressed relative to the empty expression vector pCIneo and represent the average and SD of three separate experiments. (C) 55.1c cells were transfected with expression vectors for S33Y-β-catenin and AhR together with a 3 × DRE-luciferase reporter vector and treated with 20 nM TCDD or 0.1% (v/v) DMSO as solvent control. (D) Western analysis of AhR-expression in 55.1c cells co-transfected with AhR and S33Y-β-catenin expression vectors and incubated +/- TCDD (20 nM) for 24 h.
responsiveness of 55.1c cells towards TCDD-mediated stimulation of the pT81LUC3 × DRE reporter but did not further increase S33Y-β-catenin/TCDD-mediated stimulatory effects. Incubation of hepatoma cells with TCDD results in the negative regulation of the AhR (51), an effect that was also seen with 55.1c cells in the present study (Figure 4D). We did not observe a stabilizing effect on the receptor upon transfection of cells with of S33Y-β-catenin, which essentially excludes this possibility as an explanation for the effects described above (Figure 4D).

Discussion

Alterations in xenobiotic-metabolizing enzymes are often observed during hepatocarcinogenesis. In neoplastic liver lesions, levels and activities of phase I enzymes involved in oxidative metabolism, e.g. CYPs, are lower than in normal liver tissue (5–10) (see also Figure 1A in this paper), the mechanisms mediating down-regulation of CYP expression, however, are mainly unknown. Basal CYP transcription is governed by an abundance of hepatic transcription factors, e.g. C/EBP-β, LAP and LIP, HNF-3α, HNF-4α and RXRa (52,53). A tumor-specific decrease in CYP expression may result from a change in expression or activity of key hepatocyte differentiation-associated transcription factors, which could be mediated by disturbances in cell regulatory pathways. Mouse hepatomas generated by DEN (without PB) are frequently mutated in Ha-ras (54) and display constitutive activation of the ras downstream target c-Raf kinase (55). Transfection of oncogenic Ha-ras into hepatoma cells has been shown to result in the down-regulation of CYP1A1 demonstrating that activation of a ras-dependent pathway can interfere with the expression of this enzyme (18). In contrast to phase I enzymes, detoxifying phase II enzymes, such as glutathione S-transferases and UDP-glucuronosyltransferases, are often increased in expression in neoplastic liver lesions (7,8), which together might result in reduced activation of procarcinogens and enhanced detoxification of carcinogens and several hepatotoxins and, hence, in a different susceptibility of normal and pre-neoplastic cells towards cytotoxins. It has been suggested that this ‘resistance’ confers a selective growth benefit to neoplastic cells (5,7,8,56).

Even though phase I enzymes are often negatively regulated in rodent liver foci and tumors, expression of the PB-inducible, and to a lesser extent of 3-MC-inducible isoforms can be stimulated by acute exposure of the animals to the respective inducers (7,8). This inducibility clearly demonstrates that the decrease in CYP expression seen in the absence of the inducer does not result from an irreversible block in enzyme synthesis, possibly due to alterations in the CYP encoding structural genes. In the context of the present work it is important to mention that the CYP isoforms induced in rat liver lesions by acute exposure of the animals to xenobiocides are inducer-specific, i.e. PB led to increased expression in the PB-inducible, but not in the 3-MC-inducible isoforms (7,8). In our present study mice of the respective groups were kept on a PB-containing diet until death and thus our analyses were carried out under acutely inducing conditions of the drug. It was therefore not unexpected that PB-inducible CYP2B1 isoforms were elevated in expression in liver tumors from these animals. We now observed, however, that CYP1A1 and CYP2E1, which do not constitute classical PB-inducible CYP isoforms, were also up-regulated in these tumors. In addition, we found an almost 100% correlation between expression of CYPs and expression of GS in tumors from mice of the promotion study, i.e. GS-positive lesions displayed unchanged or slightly increased CYP levels when compared with the normal surrounding tissue while GS-negative lesions generally showed decreased CYP expression. Importantly, elevated CYP levels were also seen in GS-positive lesions from mice not treated with PB. These observations suggest a common mechanism underlying induction of GS and CYPs, which may be linked to activation of β-catenin by mutation of the catnb gene in GS-positive liver tumors. This mechanism differs from the classical PB-mediated enzyme-inducing effects, which may be operative in some of the GS-negative tumors from PB-treated mice of the inhibition study, which displayed elevated expression of PB-inducible CYP isoforms.

GS was identified as β-catenin target in a genome-wide screen aimed at identifying genes over-expressed in mice expressing an activated form of the catnb gene in their liver (36). We have reported recently that GS is strongly elevated in experimentally induced mouse liver tumors harboring catnb mutations but absent in liver tumors without this mutation (37). Similarly, over-expression of GS in human HCC is strongly correlated with activated β-catenin (36). In addition to GS, several other genes are known (e.g. see http://www.stanford.edu/~russe/paths/targets.html) that are transcriptionally activated in tumors containing an activated version of β-catenin. Transcriptional regulation by β-catenin is mediated through interaction with members of the TCF/LeF1 transcription factor family (22). Interestingly, in silico analysis of the GS promoter/enhancer revealed the presence of potential TCF/LeF1-binding sites (http://corg.molgen.mpg.de/). Our present results showed that GS-positive, catnb-mutated mouse liver tumors expressed various CYP isoforms to a similar extent or at a slightly increased level when compared with normal liver tissue, whereas expression of these CYP isoforms was decreased in GS-negative, catnb wild-type tumors, both at the protein and mRNA level. To test, whether this is a direct or indirect β-catenin effect, mouse hepatoma cells were transiently transfected with an expression vector encoding activated β-catenin together with CYP2B1-Luc or 3 × DRE-Luc reporter constructs. Whereas the former was activated by β-catenin alone, the latter required the presence of TCDD for activation demonstrating that β-catenin was not capable of directly stimulating the activation of the reporter. TCDD binding to the AhR leads to nuclear translocation of the receptor, release from Hsp90 and dimerization with the partner protein Arnt, reactions required for activation of the transcription factor (4,57). The fact, that β-catenin-mediated transcriptional effects were not further enhanced by co-transfection of AhR or mCAR-1 suggests that the cellular levels of these transcription factors are either sufficiently high and therefore not rate-limiting, or, alternatively, that β-catenin-mediated stimulation of the respective CYP isoforms occurs independently of the classical nuclear induction pathways.

Genetic alterations in the Wnt/β-catenin signaling pathway are frequent in HCC and particularly in hepatoblastoma (58). In the majority of these tumors, CYPs are probably down-regulated rather than increased in expression (10), but an elevated expression of certain CYP isoforms in a subpopulation of human HCC has also been reported (59). CYP monooxygenases play a central role in the activation and/or inactivation of many anticancer drugs currently used in cancer.
therapy. Furthermore, it has been demonstrated that the multi- 
drug resistance 1 gene is transactivated by the T-cell factor 
4/β-catenin complex in colorectal carcinogenesis (60). Hence, 
β-catenin-evoked alterations in the activity of CYP enzymes 
and drug transporters might impact on tumor susceptibility 
and development of resistance towards chemotherapy.

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