Induction of UGT1A1 and CYP2B6 by an antimitogenic factor in HepG2 cells is mediated through suppression of cyclin-dependent kinase 2 activity: Cell-cycle dependent expression

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D) ABBREVIATIONS: AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; CDK, cyclin-dependent kinase; CPR, NADPH-cytochrome P450 reductase; CYP, cytochrome P450; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GR, glucocorticoid receptor; JNK, c-Jun NH2-terminal kinase; MEK, mitogen-associated protein kinase kinase; PB, Phenobarbital; PPARα, peroxisome proliferators-activated receptor α; PXR, pregnane X receptor; PCR, polymerase chain reaction; RXR, retinoid X receptor; siRNA, small interfering RNA; UGT, UDP-glucuronosyltransferase.
Abstract

Hepatocyte growth factor (HGF), an antimitogenic factor for HepG2 cells, increased mRNA and protein levels of UGT1A1 and CYP2B6 as well as the endogenous cycline-dependent kinase (CDK) inhibitors p16, p21, and p27 in HepG2 cells, but not HuH6, Caco2, or MCF7 cells. Treatment with U0126 (an ERK inhibitor) suppressed the HGF-induced expression of UGT1A1 and CYP2B6 as well as p16, p21, and p27 in HepG2 cells. The CDK inhibitor roscovitine also enhanced the expression of UGT1A1, CYP2B6, and CYP3A4. Transfection of anti-CDK2 siRNA led to elevated levels of UGT1A1, CYP2B6, and CYP3A4 in HepG2 and SW480 cells, while anti-CDK4 siRNA did not significantly enhance the expression of these enzymes. In fact, CDK2 activity was decreased in HGF-treated HepG2 cells. In cells arrested in S phase by a thymidine block and then released into a synchronous cell cycle, there was a clear dissociation among the activation of CDK2 and the expression of UGT1A1, CYP2B6, and CYP3A4. Furthermore, the induction of CYP3A4 but not UGT1A1 or CYP2B6 mRNA expression by roscovitine was repressed in pregnane X receptor (PXR) siRNA-transfected HepG2 cells. Transfection with constitutive androstane receptor (CAR) siRNA or PXR siRNA in HepG2 cells did not repress the HGF-stimulated expression of UGT1A1 mRNA. Taken together, our results demonstrate that the expression of UGT1A1 and CYP2B6 is negatively regulated through a CDK2 signaling pathway linked to cell cycle progression in HepG2 and SW480 cells, the mechanism of which may differ from that of CYP3A4 expression through PXR phosphorylated by CDK2.
Introduction

The constitutive androstane receptor (CAR, NR1I3) was originally characterized as a nuclear hormone receptor that interacts with a subset of retinoic acid response elements (Beas et al., 1994). CAR and pregnane X receptor (PXR, NR1I2) have been recognized as xenobiotic-sensing nuclear receptors that transcriptionally regulate the expression of genes of phase I, II, and III metabolic enzymes and transporters involved in the metabolism and elimination of endogenous and exogenous substances such as bilirubin, steroid hormones, and xenobiotics (Timsit and Negishi, 2007). UDP-glucuronosyltransferase, UGT1A1, plays a critical role in the detoxification of potentially neurotoxic bilirubin by conjugating it with glucuronic acid for excretion in bile (Ostrow and Murphy, 1970) and conjugates drugs and other xenobiotics (Radomska-Pandya et al., 1999; Tukey and Strassburg, 2000). We identified a phenobarbital – responsive enhancer module at -3499/-3210 from the transcription start site of UGT1A1, gtPBREM (Sugatani et al., 2001), and demonstrated that the gtNR1 (-3382/-3367) within gtPBREM plays a central role in the expression of UGT1A1 mediated by both CAR and PXR (Sugatani et al., 2005a). In addition, we demonstrated that HNF1α bound to the proximal promoter motif not only enhances the basal reporter activity of UGT1A1 including the distal (-3570/-3180) and proximal (-165/-1) regions, but also influences the transcriptional regulation of UGT1A1 by CAR, PXR, glucocorticoid receptor (GR), and arylhydrocarbon receptor (AhR) to markedly enhance reporter activities (Sugatani et al., 2008).

Previously, we demonstrated that CAR expression changes during the cell cycle in HepG2 and SW480 cells and CAR protein accumulates during G1 in both cells (Osabe et al., 2008). Here we found that UGT1A1 and CYP2B6 began to accumulate in late mitosis, preceding the CAR’s accumulation in the nucleus, and remained at high levels during G1 coinciding with CAR’s accumulation. Klinger et al. (1987) demonstrated that a transient decrease in CYPs
occurred during liver regeneration following partial hepatectomy. The hepatic growth factors, epidermal growth factor (EGF) and transforming growth factor α (TGFα), have also been demonstrated to inhibit induction of the mRNA and protein expression of the CYP2B subfamily in primary culture of mouse hepatocytes (Aubrecht et al., 1995). However, it remains to be fully elucidated how the expression of drug-metabolizing enzymes including UGT1A1 and CYP2B6 is regulated by cell signals associated with cell-cycle progression.

CAR is predominantly expressed in the liver and is found in the cytoplasm of normal mouse hepatocytes in the absence of stimuli such as drug-treatment (Kawamoto et al., 1999). CAR is activated by phenobarbital (PB) and PB-like inducers such as 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), that do not bind it directly but activate a signal transduction pathway, resulting in the translocation of CAR from the cytoplasm to the nucleus (Kawamoto et al., 1999). Koike et al. (2008) demonstrated that ERK is an endogenous signal retaining CAR in the cytoplasm in mouse primary hepatocytes, showing that (1) hepatocyte growth factor (HGF) effectively repressed the induction of endogenous Cyp2b10 gene by PB and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) in mouse primary hepatocytes, (2) HGF increased phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 in the cytosol, decreasing the TCPOBOP-induced nuclear accumulation of CAR, and (3) U0126 dephosphorylated ERK1/2 and increased nuclear CAR accumulation leading to enhanced CAR-regulated promoter activities. While HGF is a potent mitogen for hepatocytes, it is an antimitogenic factor for some tumor cell lines including HepG2; HGF has been demonstrated to induce p16 and p21 expression and decrease cyclin-dependent kinase (CDK) 2 activity, leading to inhibition of cell growth (Shima et al., 1998; Han et al., 2005). p16 and p21, which are endogenous CDK2/CDK4 inhibitors, are involved in regulating cell cycle progression. Here we found that HGF treatment increased the expression of UGT1A1 and CYP2B6 in HepG2 cells,
even when CAR levels decreased both in the cell and in the nucleus. A recent study by Lin et al. (2008) demonstrated that inhibition of CDKs by roscovitine leads to activation of PXR-mediated CYP3A4 gene expression and that CDK2 negatively regulates the activity of PXR in HepG2 cells. Thus, in the present study, we investigated the cell signaling involved in the HGF-stimulated expression of UGT1A1 and CYP2B6 in HepG2 cells in order to examine whether CDKs participate in the cell-cycle-dependent expression of UGT1A1 and CYP2B6.

**Materials and Methods**

*Materials* - U0126, U0124, LY294002, SB203580, SP600125, roscovitine, SU9516, and the CDK inhibitor p35 were purchased from Calbiochem (Darmstadt, Germany). EGF and platelet-derived growth factor (PDGF) were obtained from Sigma-Aldrich (St. Louis, MO). Histone H1 and adenosine-S-triphosphate (ATP) were from Roche Diagenostics (Mannheim, Germany). All other chemicals and solvents were of analytical grade and obtained from commercial sources.

*Cell culture conditions and treatments* - HepG2 and HuH6 human hepatoblastoma cells, Caco2 human colon carcinoma cells, and MCF7 human breast adenocarcinoma cells from RIKEN BioResource Center (Ibaraki, Japan) and SW480 human colon cancer cells from the American Type Culture Collection (1 x 10^5 cells/ml) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, HepG2, HuH6, Caco2, and MCF7 cells) and RPMI1640 medium (SW480 cells) with 10% fetal calf serum (FCS) and antibiotics (100 μg of streptomycin and 10 U of penicillin/ml) or DMEM-Ham’s F12-serum-free medium supplemented with 2 mM glutamine, 15 mM HEPES, 5 μg/ml insulin, 10 μg/ml bovine transferine, 10 ng/ml Na2SeO3, 2 μg/ml aminolevulinic acid, 25 mM glucose, 0.5 mg/ml linoleic acid-alubumin, 1 mM pyruvate with or without 100 ng/ml
human growth hormone, and the antibiotics at 37°C in the presence of 5% CO₂ unless otherwise stated.

*Cell-cycle analysis* - Cells were trypsinized and harvested at various times. They were stained with 50 μg/ml propidium iodide in flow reagent [0.1% sodium citrate, 0.2% Nonidet P-40, and 0.25 mg/ml DNase-free RNase]. Cell cycle distribution was monitored with a BD FACS Canto II flow cytometer (BD Biosciences). At least 10,000 cells were analyzed for each sample.

Short interfering RNA (siRNA)-mediated protein knockdown – siRNA targeting human CDK2 [validated stealth RNAi VHS40359 (CDK2 siRNA)]; and human PXR, 5’-aauggagaagguaguuagaaag-3’ and 5’-ccuuugacacuaccucuccauu-3’, were obtained from Invitrogen Life Technology (Carlsbad, CA, USA). siRNA targeting human CAR, 5’-gcaacugauaggaagcTdT-3’ and 5’-uucucuacucaguTdT-3’, and human CDK4, 5’-gguauccgaggaacTdT-3’ and 5’-uucucacucgguauacTdT-3’, were from B-Bridge International (Sunnyvale, CA). Cells cultured for 24h were transfected with siRNA duplexes using TransIT-siQUEST (Mirus Bio, Madison, WI) according to the manufacturer’s instructions. At 24 h after transfection, cells were given fresh medium and further transfected with the siRNA duplexes for an additional 24 h unless stated otherwise.

*Quantitative reverse transcription – PCR* – Total RNA was extracted using TRIZOL reagent from Invitrogen, and cDNAs were synthesized with a PrimeScript RT reagent kit (Takara Bio, Otsu, Japan) according to the directions. cDNA synthesized from 100 ng of total RNA was subjected to quantitative real-time PCR with Premix Ex Taq or SYBR Premix ExTaq (Takara Bio) for UGT1A1 (NM_000463), CYP1A1(NM_000499), CYP2B6 (NM_000767), CYP3A4 (NM_017460), CAR (NM_005122), PXR (NM_022002), retinoid X receptor (RXR, NM_002957), AhR (NM_001621), p16 (NM_000077), p21 (NM_000389), p27 (NM_004064), CDK2 (NM_001798), and CDK4 (NM_000075), as described previously (Osabe et al., 2009).
Western blot analysis - Treated and untreated cells were washed three times with ice-cold phosphate-buffered saline and lysed with a freshly prepared lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 1 mM NaF, 1 mM benzamidine, 1 μg/ml aprotinin, and 1 μg/ml leupeptin at 4°C. The lysates were centrifuged at 110,000 g for 10 min. Nuclear extracts were prepared using a nuclear extract kit (Active Motif). Microsomal proteins were prepared as described previously (Sugatani et al., 2004). The protein concentrations were determined by the Bradford assay (Bio-Rad). Western blotting was performed as described (Osabe et al., 2008). Briefly, nuclear extracts, microsomal proteins, or cell lysates (50 μg) were resolved on 12.5% SDS-polyacrylamide gels and the proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Milipore Corporation, Bedford, MA). Membranes were blocked at 4°C overnight in Blocking One or Blocking One-P (Nacalai Tesque, Kyoto, Japan) and probed for 1 h with primary antibodies including anti-UGT1A1, anti-CYP2B6, and anti-CYP3A4 from BD Gentest (Woburn, MA), anti-CYP1A1, and anti-CPR from Daiichi Pure Chemicals Co., Tokyo, Japan), anti-CAR (sc-13065), anti-PXR (sc-7737), anti-RXR (sc-553), anti-Histone H1 (sc-8030), anti-cyclin B1 (sc-7393), anti-cyclin D1 (sc-718) and anti-CDK4 (sc-601) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-phospho-ERK, anti-ERK, anti-p16, anti-CDK1(CDC2), anti-phospho-CDK1, anti-CDK2, anti-phospho-CDK2, and anti-β-actin from Cell Signaling Technology (Danvers, MA), anti-p21, anti-p27, and anti-cyclin A from Upstate Biotechnology (Lake Placid, NY), anti-cyclin E (Calbiochem), and anti-α-tubulin (Oncogene Research Products, Boston, MA). Antigen-antibody complexes were detected using the appropriate secondary antibody conjugated to horseradish peroxidase [horseradish peroxidase-conjugated anti-rabbit, anti-goat, or anti-mouse immunoglobulin (Jackson Immuno...
In vitro kinase assay - CDK2 kinase assays were performed as described previously (Walter et al., 2002). Cell lysates were cleared by centrifugation, and equal amounts of protein in the cell extracts (200 μg) were immunoprecipitated with 2 μg of anti-CDK2 antibody for 3 h at 4°C. Precipitated immune complexes were washed three times with the cell lysis buffer and twice with a kinase buffer [50 mM Tris-HCl, (pH 7.5) containing 10 mM MgCl₂, 1 mM dithiothreitol, 20 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, and 1 mM NaF]. Kinase reactions were performed in 10 μl of the kinase buffer containing 25 μg of histone H1, 100 μM ATP, and 0.37 MBq of [γ-32P]ATP at 30°C for 30 min. Reactions were stopped by addition of 10 μl of 2 x Laemmlli sample buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.002% bromophenol blue, and 10% 2-mecaptoethanol] and samples were resolved by SDS-PAGE on a 12.5% gel after heat denaturation. Phosphorylation of the substrate was visualized by autoradiography.

Statistics - Values are expressed as the mean ± standard error. All data were analyzed using a one-way analysis of variance. The statistical significance of differences between groups was analyzed using the ANOVA or an unpaired t-test. The level for statistically significant differences was set at p<0.05.

Results

Expression of UGT1A1, CYP2B6, and cyclin kinase inhibitors p16, p21, and p27 during HGF-induced HepG2 growth inhibition. While HGF is a potent mitogen for many types of cells

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including human hepatocytes and the tumor cell lines HuH6, Caco2, and MCF7, it is also an antimitogenic factor for several types of tumor cells such as HepG2 cells. Treatment of HepG2 cells with HGF (100 ng/ml) decreased the cell number compared to control cells (Fig. 1A). Whereas HGF downregulated the expression of CYP isozymes in human hepatocytes (Donato et al., 1998), its function in tumor cells remains to be resolved. To assess the effects of growth factor on the expression of drug-metabolizing enzymes in tumor cells, the response to HGF was compared between two opposite responding types of human hepatoblastoma cells, HepG2 and HuH6 (Shima et al., 1998) (Fig. 1B). The mRNA expression of CDK inhibitors (p16, p21, and p27) was induced 24 h after treatment with HGF in HepG2 cells, but the levels did not change in HuH6 cells. While the mRNA level of CAR decreased in HepG2 cells, the mRNA levels of UGT1A1 and CYP2B6 increased (Fig. 1B). In contrast, in Caco2 and MCF7 cells after 24 h of culture with HGF, neither the mRNA levels of UGT1A1 and CYP2B6 nor the mRNA levels of p16, p21, and p27 were increased (data not shown).

To investigate the cell signaling specifically required for stimulation by growth factors, effects of growth factors on the expression of drug-metabolizing enzymes and nuclear receptors in serum-free-medium were determined (Fig. 2). The mRNA level of UGT1A1 was increased after treatment with HGF and EGF but not PDGF, whereas the mRNA levels of CAR and PXR but not RXR and AhR were decreased (Fig. 2). The mRNA level of CYP2B6 was increased by HGF. The protein levels of UGT1A1 and CYP2B6 but not CPR were increased, whereas that of CAR in the nucleus was decreased and those of PXR and RXR remained largely unchanged after HGF-treatment (Fig. 2II and 2III). In serum-free-medium, the production of p16, p21, and p27 proteins was induced 1 h after treatment with HGF, and the expression of p21 and p27 sustained for at least 24 h, consistent with the phosphorylation of ERK1/2 (Fig. 3A). Next, to assess the signaling pathway of UGT1A1 and CYP2B6 expression after HGF treatment, we examined the
effects of U0126 (ERK inhibitor), LY294002 (PI3-kinase inhibitor), SB203580 (p38MAP kinase inhibitor) and SP600125 [c-Jun NH2-terminal kinase (JNK) inhibitor] on the expression of UGT1A1 and CYP2B6 mRNAs in cells cultured in serum-free-medium. U0126 but not U0124 (negative control) decreased the mRNA levels of UGT1A1 and CYP2B6 in HGF-treated cells (Fig. 3B), whereas the mRNA levels of CAR but not PXR, RXR, or AhR increased in the cells; the mRNA levels of CAR increased to 2.0 ± 0.3- and 1.5 ± 0.3-fold of the control by U0126 in the absence and presence of HGF, respectively (data not shown). Similarly, U0126 decreased the mRNA levels of UGT1A1 in EGF-treated cells cultured in serum-free-medium (data not shown). The HGF–induced expression of endogenous cyclin kinase inhibitors (p16, p21, and p27) was also suppressed by U0126 (Fig. 3A). These results suggest the ERK pathway to be involved in the effect of growth factors such as HGF and EGF on UGT1A1 expression as well as the induction of p16, p21, and p27 expression. On the other hand, LY294002 and SP600125 increased the mRNA levels of UGT1A1 and CYP2B6 in HGF-treated cells compared with those in the control cells, suggesting PI3-kinase and JNK to be involved in the HGF-induced expression of UGT1A1 and CYP2B6 in HepG2 cells.

Cyclin kinase inhibitors stimulate the expression of UGT1A1, CYP1A1, CYP2B6, and CYP3A4 in HepG2 cells. To explore whether cyclin kinases are involved in regulating the expression of drug-metabolizing enzymes in human cells, we examined the effects of the CDK inhibitors roscovitine [CDK1, CDK2, and CDK5 inhibitor (Meijer et al., 1999)], SU9516 [CDK1, CDK2, and CDK4 inhibitor (Yu et al., 2002)], and CDK inhibitor p35 [CDK1 and CDK2 inhibitor (Vermeulen et al., 2002)] on the expression of UGT1A1, CYP1A1, CYP2B6, and CYP3A4 mRNAs in HepG2 cells. Expression of UGT1A1 mRNA was enhanced by roscovitine, SU9516, and CDK inhibitor p35 in a dose-dependent manner (Fig. 4A). Roscovitine markedly stimulated the expression of UGT1A1 mRNA and protein, while it slightly stimulated the
expression of CYP1A1, CYP2B6, and CYP3A4 mRNAs and proteins (Fig. 4). Overall, the findings indicated that CDKs were clearly involved in regulating the expression of UGT1A1 and CYP2B6 in HepG2 cells.

**Effects of anti-CDK4 siRNA and anti-CDK2 siRNA on induction of UGT1A1 and CYP2B6 expression.** Next, to determine whether CDK4 and CDK2, the activities of which were inhibited by p16, p21, and p27, were required for the downregulation of UGT1A1 and CYP2B6 expression, anti-CDK4 siRNA or anti-CDK2 siRNA was introduced into HepG2 and SW480 cells. As shown in Figure 5, transfection with the anti-CDK4 siRNA dramatically reduced the levels of CDK4 mRNA and protein in HepG2 cells, but was not followed by an increase in mRNA and/or protein levels of UGT1A1, CYP1A1, CYP2B6, and CYP3A4. In contrast, transfection with the anti-CDK2 siRNA in HepG2 and SW480 cells was followed by an increase in mRNA levels of UGT1A1, CYP2B6, and CYP3A4 (Fig. 6). Furthermore, transfection with the anti-CDK2 siRNA led to stimulated expression of UGT1A1, CYP2B6, and CYP3A4 proteins but not CYP1A1 protein in HepG2 and SW480 cells as well as a decrease in CDK2 protein levels, with the extent of the stimulation by anti-CDK2 siRNA greater in SW480 cells than in HepG2 cells (Fig. 7).

To determine whether the CDK2 activity was reduced in HepG2 cells after treatment with HGF, we analyzed the extent of phosphorylation of Histone H1 after incubation with the cell lysate. As shown in Figure 8, the extent of phosphorylation was decreased in HGF-treated cell lysate compared with control lysate, indicating that the CDK2 activity was actually decreased in the HGF-treated HepG2 cells, and that CDK2 activity was required for downregulation of UGT1A1, CYP2B6, and CYP3A4 expression.

**CDK2 is involved in the cell-cycle-dependent downregulation of UGT1A1, CYP2B6, and CYP3A4.** To investigate whether the expression of UGT1A1, CYP2B6, and CYP3A4 changes
during the cell cycle, we undertook a detailed analysis of protein levels in synchronously dividing SW480 cells. SW480 cells arrested in S-phase by a double-thymidine block were released into a synchronous cell cycle and sampled every 2 h. Cells completed S-phase in 6 h of their release from the thymidine block and mitosis in 10 h (Fig. 9). Protein levels of cyclin kinases, cyclins, and drug-metabolizing enzymes in the cell lysate were determined by Western blotting (Fig. 9). As described in the previous study (Osabe et al., 2008), cyclin A and cyclin B1 protein levels decreased at 12 h to 16 h after release from the thymidine block as cells exited G1. Cyclin D1 was present at very low levels at 0 h after the release and started accumulating. The protein levels of cyclin E1 remained unchanged. CDK4 was present at 0 h to 6 h after release from the thymidine block and started accumulating at 10 h. We found that while the total protein levels of CDK1 and CDK2 remained largely unchanged in the cells after release from the thymidine block for 16 h, the active form of CDK2 (phospho-CDK2) was increased and peaked at 2 h, followed by the active form of CDK1 (phospho-CDK1), which peaked at 4 h. The time-dependent changes in the phospho-CDK2 level matched the time-dependent accumulation of S-phase cells. While the active form of CDK2 (phospho-CDK2) was present at high levels at 2 h to 6 h after release from the thymidine block and these levels dropped at 8 h, UGT1A1 and CYP2B6 were present at very low levels at 0 h to 6 h and started accumulating at 8 h. CYP3A4 was present at very low level at 0 h to 4 h after release from the thymidine block and started accumulating at 6 h, while the protein levels of CPR remained largely unchanged. Moreover, similar cell-cycle-dependent expression of UGT1A1, CYP2B6, and CYP3A4 in HepG2 cells was found as in SW480 cells (data not shown).

Regulatory mechanism of HGF- and roscovitine-stimulated UGT1A1 and CYP2B6 gene expression was different from that of the CYP3A4 gene expression. Recently, Lin et al. (2008) reported that CDK2 directly phosphorylates PXR and negatively regulates human PXR-mediated
CYP3A4 gene expression in HepG2 cells. To determine whether the HGF- and roscovitine-stimulated UGT1A1 and CYP2B6 gene expression is regulated through a pathway mediated by CAR or PXR as well as CYP3A4 gene expression, anti-CAR siRNA or anti-PXR siRNA was introduced into HepG2 cells. As shown in Figure 10A and 10B, transfection with anti-CAR siRNA and anti-PXR siRNA reduced the levels of CAR and PXR mRNAs in HepG2 cells, respectively, but did not change the mRNA expression of UGT1A1 stimulated by HGF. Transfection with anti-CAR siRNA did not change the mRNA levels of UGT1A1 and CYP3A4 stimulated by roscovitine (Supplemental Fig. 2). Furthermore, whereas transfection with anti-PXR siRNA reduced the mRNA levels of CYP3A4 stimulated by roscovitine, mRNA levels of UGT1A1 and CYP2B6 after treatment with roscovitine remained similar to those in control cells (Fig. 10C). These findings suggest that the mechanism to downregulate the expression of UGT1A1 and CYP2B6 gene by HGF and roscovitine may differ from that of CYP3A4 gene.

Discussion

Reduced bilirubin glucuronosyltransferase (UGT1A1) activity is associated with the development of unconjugated hyperbilirubinemia (Crigler-Najjar syndrome and Gilbert’s syndrome) (Mackenzie et al., 1997) and increased side effects of drug treatment such as the predisposition of patients to toxicity initiated by SN-38, an active metabolite of the anticancer drug irinotecan (Gange et al., 2002; Tukey et al., 2002). Understanding the molecular mechanisms of the induction of human UGT1A1 may provide information for the prevention and treatment of unconjugated hyperbilirubinemia and the side effects of drugs. Whereas the proximal 165-bp promoter motif is regulated by HNF1α (Bernard et al., 1999), the distal 290-bp enhancer module (gtPBREM) of human UGT1A1 is regulated by CAR as a transcription factor in response to phenobarbital treatment (Sugatani et al., 2001), by PXR as a nuclear receptor.
responsible for rifampicin-induced activation of gtPBREM (Sugatani et al., 2004), by GR as a nuclear hormone receptor capable of activating gtPBREM by dexamethasone (Sugatani et al., 2005b), by the receptor-type transcription factor AhR responsible for \( p \)-naphthoflavone-induced activation (Yueh et al., 2003), and by peroxisome proliferators-activated receptor \( \alpha \) (PPAR\( \alpha \)) responsible for WY-14643 (Seneko-Effenberger et al., 2007). The 290-bp gtPBREM is characterized as a composite regulatory element containing the multiple binding sites DR4, gtNR1, DR3, glucocorticoid-response elements (GRE1 and GRE2) for the nuclear receptors CAR, PXR and GR (Sugatani et al., 2001, 2004, 2005b), as well as the AhR response element (Yueh et al., 2003) and the PPAR\( \alpha \) response element (Seneko-Effenberger et al., 2007). Notably, the nuclear receptor CAR is essential for regulating UGT1A1 and the CYP2B subfamily by PB and PB-like inducers (Sugatani et al., 2005a; Timsit and Negishi, 2007).

Hepatocyte growth factor (HGF) is known not only as a potent mitogen for hepatocytes and several types of tumor cells including HuH6, Caco2, and MCF7 cells, but also as an anti-mitogenic factor for some types of tumor cells such as HepG2 cells. In regenerating rat liver induced by extended hepatectomy, the expression of liver-specific genes such as the \textit{serum albumin} and \textit{CYP2B} genes has been demonstrated to be suppressed (Kakizaki et al., 2007). Moreover, Thasler et al. (2006) demonstrated that an augmenter of liver regeneration, HGF, reduced CAR but not PXR or AhR expression and downregulated basal and induced CYP expression in human hepatocytes in vitro, indicating that growth signals influence hepatic drug metabolism. Downregulation of CYP expression by related factors such as EGF and TGF\( \alpha \) has been also observed in humans (Greuet et al., 1997). In contrast, this study demonstrated that HGF stimulated the expression of UGT1A1 and CYP2B6 in HepG2 cells whose growth was suppressed by it, whereas HGF did not stimulate the expression of these enzymes in HuH6 cells.
While EGF also stimulated the expression of UGT1A1 in HepG2 cells, it has been demonstrated that the receptor of HGF, c-Met, associates with EGF receptor in human hepatoma cell lines including HepG2 and this association facilitates the phosphorylation of c-Met in the absence of HGF (Jo et al., 2000). Shima et al. (1998) found that (1) protein levels of p21 markedly decreased in HGF-treated HuH6 cells, but high levels of p21 were sustained for 24 h after HGF treatment in HepG2 cells, and (2) significant levels of p16 and p27 were observed after 24 h only in HepG2 cells. The induction of p16, p21, and p27 expression by HGF in HepG2 cells is considered to lead to growth inhibition by inhibiting CDKs (CDK2 and CDK4) as reported previously (Shima et al., 1998; Han et al., 2005). Here we found that (1) inhibition by U0126 of HGF downstream of MEK led to the repression of expression of both CDK inhibitors (p16, p21, and p27) and the drug-metabolizing enzymes UGT1A1 and CYP2B6 in spite of upregulating CAR expression in HepG2 cells (Figs. 1-3), (2) HGF did not induce the expression of UGT1A1 and CYP2B6 in HuH6 cells, which did not follow the induction of endogenous CDK inhibitors (Fig. 1), and (3) the CDK inhibitor roscovitine stimulated the expression of UGT1A1 and CYP2B6 in HepG2 cells (Fig. 4). The findings suggested the endogenous CDK inhibitors to be involved in the HGF-induced expression of UGT1A1 and CYP2B6 in HepG2 cells (Fig. 11).

In the regulatory network of progression through the mammalian cell-cycle, cyclin D-CDK4 is activated at G1, cyclin E-CDK2 is necessary for G1/S transition, and the activation of CDK4 and CDK2 leads to the nuclear accumulation of cyclin-CDK complexes during G1 (Jaumot et al., 1999). As shown in Figure 11, cyclin D1/CDK4/CDK6 and cyclin E/CDK2 complexes cooperate in RB phosphorylation, leading to its inactivation, dissociation of the transcription factor E2F and cell cycle progression (Lundberg et al., 1999). Thus, to investigate whether CDK plays a key role in the induction of UGT1A1 and CYP2B6 expression, we...
examined the effect of CDK inhibitors in HepG2 cells. We showed that the expression of UGT1A1 mRNA was induced by roscovitine, SU9516, and CDK inhibitor p35, which all inhibit CDK1 and CDK2 activities (Fig. 4). Since p16, p21, and p27 repress CDK2 and CDK4 activities, the induction of UGT1A1 expression by roscovitine may be due to the inhibition of CDK2 activity. To confirm whether CDK2 contributes to the induction of UGT1A1 and CYP2B6 expression through cell cycle progression, we deleted CDK2 using siRNA in HepG2 cells expressing the HGF receptor and SW480 cells expressing no HGF receptor (Zeng et al., 2004). CDK2 deletion resulted in the expression of UGT1A1 and CYP2B6 in both cells, whereas CYP1A1 protein levels were not largely changed in CDK2-deleted cells (Figs. 6 and 7). In fact, the level of CDK2 activity in HGF-treated cells was decreased (Fig. 8). CDK2, a key regulator of G1-S cell cycle progression, is activated by Akt-mediated phosphorylation (Maddika et al., 2008). The inhibition by LY294002 of HGF downstream of the PI3 kinase-Akt signaling pathway led to an increase in the expression of UGT1A1 and CYP2B6 (Fig. 3), also suggesting the involvement of CDK2 in the HGF-induced expression of UGT1A1 and CYP2B6 in HepG2 cells (Fig. 11). In contrast, CDK4 deletion using siRNA in HepG2 cells did not lead an increase in the expression of UGT1A1 and CYP2B6 (Fig. 5). Furthermore, there was a clear dissociation among the expression of activated CDK2 and that of UGT1A1, CYP2B6, and CYP3A4 in cells arrested in S phase by a double-thymidine method and then released into a synchronous cell cycle (Fig. 9). The findings suggested activated CDK2 to be essential for downregulating the expression of UGT1A1 and CYP2B6. In addition, the inhibition by SP600125 of HGF downstream of the JNK signaling pathway also increased the expression of UGT1A1 and CYP2B6 (Fig. 3). Since Lu et al. (2009) demonstrated that docosahexaenoic acid downregulates PB-induced CYP2B1 gene expression in rat primary hepatocytes via the JNK pathway, JNK may be involved in regulating the expression of UGT1A1 and CYP2B6 by HGF.
Does CAR or PXR play an essential role in UGT1A1 and CYP2B6 expression in HGF-stimulated HepG2 cells? The CAR protein level not only in the cell lysate (Osabe et al., 2008) but also in the nucleus (Fig. 2) was reduced after HGF treatment. In addition, the suppression of CAR expression using anti-CAR siRNA did not repress the HGF- and roscovitine-induced expression of UGT1A1 (Fig. 10). These observations indicate that changes in the expression of CAR did not affect UGT1A1 levels in HGF- and roscovitine-treated HepG2 cells. A recent study by Lin et al. (2008) demonstrated that CDK2 directly phosphorylates PXR and negatively regulates the activity of PXR, leading to the repression of PXR-mediated CYP3A4 expression by roscovitine. However, in the present study, although PXR deletion using siRNA repressed the roscovitine-stimulated CYP3A4 expression in HepG2 cells, it did not affect the HGF- or roscovitine-stimulated expression of UGT1A1 and CYP2B6 (Fig. 10). These observations suggest that CDK2 negatively regulates the expression of UGT1A1 and CYP2B6 as well as CYP3A4, but the factor(s) phosphorylated by CDK2, which is involved in the UGT1A1 and CYP2B6 gene expression, may be different from PXR.

Does the cell-cycle-dependent expression of drug-metabolizing enzymes in human tissues have any implications? When treating patients with drugs modulating cell cycle progression such as the anticancer agent E7070, which arrests cells at the G1/S boundary (Van den Bongard et al., 2004), the pharmacokinetics of other therapeutic drugs may be influenced, resulting in side effects. One has to pay attention to drug-drug interactions under such conditions. Since in HepG2 and SW480 cells, CDK2 deletion using siRNA led to an increase in the gene expression of UGT1A1 and CYP2B6 (Fig. 6), the CDK2 signaling pathway may play an essential role in the induction of UGT1A1 and CYP2B6 expression in human cells including hepatocytes. It remains to be clarified how the expression of UGT1A1 and CYP2B6 is modulated by CDK2 and
JNK through the cell cycle. We are now studying the molecular mechanism by which UGT1A1 and CYP2B6 expression is negatively regulated by CDK2 and JNK in human tumor cells and normal cells.
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Footnote

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Legends for figures

Fig. 1  Effect of HGF on cell growth (A) and expression of UGT1A1, CYP2B6, CAR, PXR, RXR, p16, p21, and p27 (B) in HepG2 and HuH6 cells. Cells cultured for 24 h were treated with HGF (100 ng/ml) or vehicle (0.1% BSA in saline) for 96 h in HepG2 cells (A) or for 24 h in HepG2 and HuH6 cells (B). In B, the mRNA levels in cells cultured for 24 h were taken as 1.0. Data represent means ± S.E. for 3 experiments. * P<0.05, ** P< 0.01, *** P< 0.001, versus vehicle-treated control group.

Fig. 2  Effect of growth factors on expression of UGT1A1, CYP2B6, CAR, PXR, RXR, and AhR in HepG2 cells. Cells cultured for 48 h were placed in 10% FCS (A)- or 0.1% FCS (B-E)-supplemented medium for 24 h and then cultured with 10% FCS-supplemented medium (A) or serum-free-medium (B-E) and treated with vehicle (0.1% BSA in saline (B), HGF (100 ng/ml, C), EGF (100 ng/ml, D), and PDGF (100 ng/ml, E) for an additional 24 h. In I, the mRNA levels in cells cultured for 48 h were taken as 1.0. Data represent means ± S.E. for 3 experiments. * P<0.05, ** P< 0.01, *** P< 0.001. In II and III, protein levels in the microsome fractions (II) and nuclear extract (III) were determined by Western blotting with the indicated antibodies. Band intensities were measured with NIH Image software, and numbers in parentheses indicate relative levels, which were normalized with the protein levels in control cells (II, B group; III, A group).

Fig. 3  Effect of cell signaling inhibitors on phosphorylation of ERK (A), expression of p16, p21, and p27 proteins (A), and expression of UGT1A1 and CYP2B6 mRNAs (B) in HGF-treated HepG2 cells. Cells cultured for 48 h were replaced with DMEM-Ham’s
F12-serum-free-medium and treated with various inhibitors (10 μM) or vehicle (dimethylosulfoxide) in the presence (closed bar) or absence (open bar) of HGF (100 ng/ml)) for an additional 24 h. In A, HepG2 cells cultured for 48 h were placed in serum-free-medium and cultured with HGF (100 ng/ml) in the absence and presence of 10 μM U0126. Whole-cell lysate was then obtained at 0.3, 1, 3, 6, and 24 h after treatment, and phospho-ERK1/2 and ERK1/2 protein levels were determined by Western blotting. In B, the mRNA levels in cells cultured for 48 h (control group) were taken as 1.0. Data represent the means ± S.E. for 3 experiments. **p< 0.01, ***p< 0.001 versus cells cultured in the absence of HGF.

Fig. 4 Effect of CDK inhibitors on expression of UGT1A1, CYP1A1, CYP2B6, and CYP3A4 in HepG2 cells. Cells cultured for 48 h were replaced with 10% FCS-supplemented medium and treated with various inhibitors or vehicle (dimethylosulfoxide) for an additional 24 h. In A and B, the mRNA levels in vehicle-treated cells were taken as 1. Data represent the means ± S.E. for 3 experiments. *p<0.05, **p<0.01, ***p<0.001 versus vehicle-treated cells. In C, protein levels in whole-cell lysate after treatment with roscovitine (5 μM) or vehicle were determined by Western blotting.

Fig. 5 Effect of anti-CDK4 siRNA transfection on expression of CDK4, CDK2, UGT1A1, CYP1A1, CYP2B6, and CYP3A4 in HepG2 cells. HepG2 cells cultured for 48 h were transfected with anti-CDK4 siRNA (100 nM) or mock. At 48 h after transfection, cells were collected and relative mRNA (A) and protein (B) levels were determined by quantitative real-time PCR and Western blotting, respectively. The mRNA levels in
cells cultured for 48 h (control group) were taken as 1. Data represent the means ± S.E. for 3 experiments. ***p<0.001 versus mock group.

Fig. 6 Effect of anti-CDK2 siRNA transfection on expression of CDK2, CDK4, UGT1A1, CYP1A1, CYP2B6, and CYP3A4 mRNAs in HepG2 and SW480 cells. HepG2 (A) and SW480 (B) cells cultured for 24 h were transfected with anti-CDK2 siRNA (100 nM) or mock. At 24 h after transfection, cells were replaced with each medium, further transfected with anti-CDK2 siRNA or mock, and then cultured for an additional 24 h. The mRNA levels in cells cultured for 48 h (control group) were taken as 1. Data represent the means ± S.E. for 3 experiments. **p<0.01, ***p<0.001 versus mock group.

Fig. 7 Effect of anti-CDK2 siRNA transfection on expression of CDK2, CDK4, UGT1A1, CYP1A1, CYP2B6, CYP3A4, CAR, PXR and β-action proteins in HepG2 (A) and SW480 (B) cells. In A and B, at 48 h after transfection as shown in Fig. 6, cells were collected and protein levels in the whole-cell lysate were determined by Western blotting.

Fig. 8 Effect of HGF on the kinase activity of CDK2 in HepG2 cells. Cells cultured for 24 h were placed with 10% FCS-supplemented medium and treated with vehicle (0.1% BSA in saline or HGF (100 ng/ml) for an additional 24 h. Whole-cell lysate was prepared as shown in Figure 3A and immunoprecipitated (IP) with anti-CDK2 antibody. Immune complex kinase assays were performed using Histone H1 as a substrate. Experiments were done twice with similar results, and representative data are shown.
Fig. 9 Cell-cycle-dependent expression of UGT1A1 and CYP2B6. SW480 cells arrested in S-phase by a double-thymidine method were released into a synchronous cell cycle and sampled every 2 h. Double thymidine block was done by incubating the cells at day 2 in DMEM-10% FCS containing 2 mM thymidine overnight. The cells were then washed and incubated in fresh DMEM-10% FCS for 8 h. A second overnight incubation in 2 mM thymidine was done before washing and releasing the block in DMEM-10% FCS. DNA content was analyzed with a FACS Canto II flow cytometer while protein levels in the cell lysate at the indicated times (hours after release from S) were determined by Western blotting with the indicated antibodies.

Fig. 10 Effect of anti-CAR siRNA and anti-PXR siRNA transfection on expression of UGT1A1, CYP2B6, and CYP3A4 in HepG2 cells treated with HGF or roscovitine. HepG2 cells cultured for 24 h were transfected with anti-CAR siRNA (50 nM), anti-PXR siRNA (100 nM) or mock. At 24 h after transfection, cells were replaced with each medium, further transfected, and then cultured with HGF (100 ng/ml, closed bar) or vehicle (0.1% BSA in saline, open bar), or roscovitine (5 μM, hatched bar) or vehicle (dimethylsulfoxide, open bar) for an additional 24 h. The mRNA levels in cells cultured for 48 h (control group) were taken as 1. Data represent the means ± S.E. for 3 experiments. +p=0.06, *p<0.05, **p<0.01, ***p<0.001 versus vehicle-treated control group; #p<0.05 anti-PXR siRNA-transfected and HGF- or roscovitine-treated group versus HGF- or roscovitine-treated control group.

Fig. 11 Schematic model of the signaling pathway involving CDK2 and CDK inhibitors (p16,
p21, and p27) and induction of UGT1A1 and CYP2B6 during HGF-induced growth inhibition of HepG2 cells is presented.

Supplemental Fig. 1

The proteins in human CYP3A4-expressed microsomes (BD Gentest, Woburn, MA) were detected by Western blotting with anti-CYP3A4 antibody (BD Gentest).

Supplemental Fig. 2

Effect of anti-CAR siRNA transfection on expression of UGT1A1 and CYP3A4 in HepG2 cells treated with roscovitine. The cells were treated as shown in Figure 10. The mRNA levels in cells cultured for 48 h (control group) was taken as 1. Data represent the means ± S.E. for 3 experiments. *p<0.05, **p<0.01, ***p<0.001 versus vehicle-treated control group.
Fig. 1

(A) Graph showing cell number (×10^5) over time (h) with and without HGF.

(B) Bar charts showing fold induction for UGT1A1, CYP2B6, CAR, p16, p21, and p27.

- **UGT1A1**
  - HepG2: + HGF: 3.0, - HGF: 1.0
  - HuH6: + HGF: 2.0, - HGF: 1.0

- **CYP2B6**
  - HepG2: + HGF: 5.0, - HGF: 1.0
  - HuH6: + HGF: 5.0, - HGF: 1.0

- **CAR**
  - HepG2: + HGF: 4.0, - HGF: 1.0
  - HuH6: + HGF: 1.0, - HGF: 1.0

- **p16**
  - HepG2: + HGF: 4.0, - HGF: 1.0
  - HuH6: + HGF: 1.0, - HGF: 1.0

- **p21**
  - HepG2: + HGF: 2.0, - HGF: 1.0
  - HuH6: + HGF: 1.0, - HGF: 1.0

- **p27**
  - HepG2: + HGF: 3.0, - HGF: 1.0
  - HuH6: + HGF: 1.0, - HGF: 1.0

**Notes:**

- ***: p < 0.001
- **: p < 0.01
- *: p < 0.05

**Legend:**

- -: Control
- +: Treatment
Fig. 2
Figure 3

(A) Western blot analysis of phospho-ERK1/2, ERK1/2, p16, p21, p27, and α-Tubulin under HGF(-) and HGF(+) conditions. Time points are 0, 0.3, 1, 3, 6, and 24 hours.

(B) Bar graphs showing the fold induction of UGT1A1 and CYP2B6 under various treatments: Control, Vehicle, U0126, LY294002, SB203580, and SP600125. The data are represented with error bars and significance levels indicated by asterisks: *** for p < 0.001 and ** for p < 0.01.
Fig. 4
(A) 

![Graph showing fold induction for different genes with Mock and CDK4 siRNA treatments.](graph.png)

(B) 

![Western blot images for CDK4, CDK2, UGT1A1, CYP1A1, CYP2B6, CYP3A4, and β-Actin under Mock and CDK4 siRNA conditions.](blot.png)

Fig. 5
(A) HepG2 cells

- Mock
- CDK2 siRNA

Fold induction

CDK2  CDK4  UGT 1A1  CYP 1A1  CYP 2B6  CYP 3A4

***  ***  5  10  15  20

(B) SW480 cells

- Mock
- CDK2 siRNA

Fold induction

CDK2  CDK4  UGT 1A1  CYP 1A1  CYP 2B6  CYP 3A4

***  ***  5  10  15  20

Fig. 6
![Western Blot Image]

**Day 2**

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**Fig. 9**