The human pregnane X receptor (hPXR) regulates the expression of critical drug metabolism enzymes. One of such enzymes, cytochrome P450 3A4 (CYP3A4), plays critical roles in drug metabolism in hepatocytes that are either quiescent or passing through the cell cycle. It has been well established that the expression of P450, such as CYP3A4, is markedly reduced during liver development or regeneration. Numerous studies have implicated cellular signaling pathways in modulating the functions of nuclear receptors, including hPXR. Here we report that inhibition of cyclin-dependent kinases (Cdks) by kenpaullone and roscovitine — two small-molecule inhibitors of Cdks that we identified in a screen for compounds that activate hPXR — leads to activation of hPXR-mediated CYP3A4 gene expression in HepG2 human liver carcinoma cells. Consistent with this finding, activation of Cdk2 attenuates the activation of CYP3A4 gene expression. In vitro kinase assays revealed that Cdk2 directly phosphorylates hPXR. A phosphomimetic mutation of a putative Cdk phosphorylation site, serine350, significantly impairs the function of hPXR, while a phosphorylation-deficient mutation confers resistance to Cdk2. Using HepG2 that has been stably transfected with hPXR and the CYP3A4-luciferase reporter, enriched in different phases of the cell cycle, we found that hPXR-mediated CYP3A4 expression is greatly reduced in the S phase. Our results indicate for the first time that Cdk2 negatively regulates the activity of hPXR, and suggest an important role for Cdk2 in regulating hPXR activity and CYP3A4 expression in hepatocytes passing through the cell cycle, such as those in fetal or regenerating adult liver.

Numerous studies have implicated signaling pathways in the modulation of activities of nuclear receptors (NRs); a superfamily of ligand-activated transcription factors (1-5). For example, both protein kinase A (PKA) and protein kinase C (PKC) have been shown to modulate the activity of the pregnane X receptor (PXR), which is a member of the NR superfamily (6,7). The human PXR (hPXR) is also referred to as a “steroid and xenobiotic receptor,” or SXR (8-10). PXR is a key xenobiotic receptor regulating the metabolism and excretion of both xenobiotics and endobiotics by regulating the expression of drug metabolizing enzymes and drug transporters (1,11). This regulation is achieved through the binding of hPXR to its xenobiotic response elements present in the promoter regions of the drug metabolizing enzymes and transporters.

One extremely important hPXR-target gene is that of cytochrome P450 3A4 (CYP3A4). CYP3A4 catalyzes the metabolism of over 50% of all clinically-prescribed drugs (12). Changes in the expression of CYP3A4 can affect drug metabolism, thereby altering the therapeutic or toxicological response to a drug, and possibly causing other adverse drug interactions; an important clinical problem representing a major contributor to morbidity and mortality. A plethora of structurally-diverse molecules, including therapeutic drugs, directly bind to and activate hPXR to induce the expression of CYP3A4 (1). Additionally, like other NRs, hPXR can be modulated by cell signaling pathways. Thus, the activity of hPXR is not only regulated by ligands, but also by cell signaling pathways such as the protein kinase pathways.

Among the kinases that regulate NRs are the cyclin-dependent kinases (Cdks), which recognize and phosphorylate a serine (Ser) or threonine (Thr) residue followed by a proline (Pro) (13,14). For
example, the activity of Cdk2 is required for the function of the progesterone receptor (PR) (15), and Cdk1 has been shown to phosphorylate and stabilize the androgen receptor (AR) (3). The activity of a Cdk requires a cyclin, which serves as a regulatory subunit for the Cdk. Different cyclin-Cdk complexes drive the cell cycle through its different phases (i.e., G1, S, G2, and M) in response to different signals, such as mitogenic signals (16). For example, during liver regeneration, growth factors such as hepatocyte growth factor (HGF) and epidermal growth factor (EGF) can drive the cell cycle to pass the G1/S checkpoint and stimulate DNA synthesis (17).

hPXR and CYP3A4 are primarily expressed in liver and intestine. Hepatocytes in normal adult liver are quiescent (G_0 phase) and exhibit only minimal response to mitogens (17). However, loss of liver mass due to chemical, traumatic, or infectious liver injuries can trigger a regenerative response in adult livers. Liver regeneration is mainly achieved through driving the quiescent mature adult hepatocytes to re-enter the cell cycle from the G_0 phase (18,19).

Interestingly, it has been reported that CYP3A4 expression is reduced during liver regeneration (20), although the mechanism responsible for this reduction is unclear. In addition, there is a high rate of hepatocyte proliferation during liver development (21). Significant changes in the expression of the cytochrome P450 family, including CYP3A4, occur during liver development (22,23). The CYP3A4 level is extremely low in fetal liver, and progressively increases shortly after birth. Reduction in drug metabolism enzymes, including CYP3A4, might have a profound effect on therapeutic efficacy and the risk of adverse drug reactions in the fetus and child, as well as adult patients with regenerating liver due to various liver injuries. However, the reason why hepatocytes passing through the cell cycle have lower CYP3A4 levels than quiescent hepatocytes remains unclear.

Moreover, hPXR expression has been detected in prostate cancer (24), endometrial cancer (25), and osteosarcoma (26), suggesting a role for PXR in these actively dividing human cancer cells.

Since understanding the molecular mechanisms responsible for changes in enzymes involved in drug metabolism is important for designing effective therapies that prevent adverse drug interactions, and since cellular signaling pathways have been implicated in modulation of NR activities, we sought a cell-based screening approach that would identify compounds that activate hPXR-mediated gene expression. By screening a library of known bioactive compounds for small molecule hPXR activators, we identified two Cdk inhibitors — kenpaullone and roscovitine — that strongly activate the hPXR signaling pathway but only weakly bind to hPXR. Consistent with this observation, we show that activation of Cdk2 leads to the attenuation of hPXR activity. In addition, we show that Cdk2 directly phosphorylates hPXR in vitro, and that a phosphomimetic mutation of a putative Cdk phosphorylation site, Ser<sup>350</sup>, significantly impairs the function of hPXR, while a phosphorylation-deficient mutation confers resistance to the inhibitory effect of Cdk2. We also provide evidence that inhibition of hPXR activity occurs in the S phase of the cell cycle. Our data suggests that the activity of hPXR is negatively regulated by Cdk2. To our knowledge, this is the first report that links the activity of hPXR to Cdns and the cell cycle.

**Experimental Procedures**

**Materials-** HepG2 liver carcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). G418 and other cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Anti-V5 was obtained from AbCam (Cambridge, MA). Anti-FLAG M2, anti-β-actin, DMSO, histone H1, thymidine, kenpaullone, rifampicin, and SR-12813 were obtained from Sigma (St. Louis, MO). Roscovitine was purchased from EMD Chemicals (Gibbstown, NJ). Purified Cdk2/cyclin E complex was obtained from Millipore (Billerica, MA). Purified Cdk2/cyclin A complex was obtained from New England Biolabs (Ipswich, MA). Purified hPXR protein was obtained from Origene Tech (Rockville, MD). γ<sup>32</sup>-ATP was purchased from Perkin Elmer (Waltham, MA). Alamar blue was purchased from BioSource (Camarillo, CA). Charcoal/dextran-treated FBS was purchased from Hyclone (Logan, UT).

**Cell culture, plasmids, and transfection-** All cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. HepG2 was maintained in modified Eagle’s minimal essential medium from ATCC with 10% FBS, 2 mM L-glutamine, and 1% pen/strep. The pcDNA3-<i>hPXR</i> construct was prepared following a method described previously (27). The FLAG-<i>hPXR</i>, a construct expressing a FLAG-tagged hPXR with the FLAG epitope (N-DYKDDDDK-C) fused to the N-terminus of hPXR, was prepared by
subcloning a fragment containing hPXR into pcDNA3-FLAG. The pcDNA3-FLAG was prepared by annealing oligonucleotides 5'-AGCTGCCACCATGACTACAAGGACGACGATGACAAGGACCA-3' and 5'-AGCTTGGTCCTTGTACGTGTCCTTGTAGTCCATGGTGGC-3' and ligating the resulting fragment into HindIII-cleaved pcDNA3 (Invitrogen). Plasmids for amino acid substitution mutants of hPXR (FLAG-hPXR S350A and FLAG-hPXR S350D) were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and appropriately-mutated primers. Mutations were verified through nucleotide sequencing. The CYP3A4-luciferase reporter was constructed in pGL3 (Promega, Madison, WI) following a method described previously (28). The resulting reporter, designated CYP3A4-luc, contained the following CYP3A4 promoter regions: -7836 to -7208 and -362 to +53. Cyclin A construct was a gift from Dr. Nancy Weigel. V5-cyclin E and V5-Cdk2 constructs were gifts from Dr. Haojie Huang. CMV-Renilla luciferase plasmid was obtained from Promega. Transfections were performed using FuGEN6 (Roche Diagnostics, Indianapolis, IN) accordingly to the manufacturer’s instructions. For transient transfection, HepG2 cells were first transfected. Forty-eight hours post-transfection, the cells were treated with compounds for an additional 24 h before luciferase assay. To select stable clones, the transfected cells were selected in medium containing 800 µg/mL of G418 and maintained in medium containing 400 µg/mL of G418. Single colonies were isolated, expanded, and tested for expression of hPXR and response to rifampicin. One of such stable clones, clone 1, was used in this study.

**hPXR transactivation assay**- The methodology, as previously reported, was followed with minor modifications (29). Briefly, compounds were added to the wells of 384-well plates containing either transiently transfected or stable HepG2 cells (final DMSO concentration was 0.1%) in phenol red–free medium containing 5% charcoal/dextran-treated FBS, and incubated for 6 or 24 h at 37°C before luciferase assay. DMSO was used as a negative control for compound treatment. In the compound screening (see below for details), rifampicin (10 µM), a well known hPXR agonist, was used as a positive control. The data was expressed as a percentage of activation (% Act = 100% x [compound signal – DMSO signal]/[10 µM rifampicin signal – DMSO signal]). Curve-fitting software (GraphPad Prism 4.0; Graphpad Software, La Jolla, CA) was used to generate the curves and to determine the EC_{50} values. Consistent with data previously reported (30), rifampicin had an EC_{50} of ~1.3 µM in this assay.

In the transient transfection studies, a CMV-Renilla plasmid was co-transfected and the activities of the reporters were measured using the Dual-glo luciferase kit from Promega, accordingly to the manufacturer’s instructions. HepG2 cells in a T-25 tissue culture flask containing ~3 million cells (70% to 80% confluency) were transfected with 2.5 µg of total plasmids. To prepare the plasmid mix, 0.3 µg of CMV-Renilla, 1.9 µg of CYP3A4-luc, 0.1 µg of FLAG-hPXR, 0.5 µg of V5-Cdk2 and/or cyclin constructs (V5-cyclin E or cyclin A) were mixed with 9 µL of FuGEN6 diluted in 300 µL of serum-free medium. Then, 250 µL of the mixture was used to transfect HepG2 cells in a T-25 flask. Where Cdk2 or cyclin constructs were not used, a pcDNA3 vector was used to bring the total amounts of plasmid to 3.3 µg. At 24 h post-transfection, 5,000 transfected cells per well (25 µL per well) were seeded into 384-well tissue culture–treated solid white plates. Twenty-four hours later, the cells were treated with compounds for another 24 h prior to luciferase assay. Relative luciferase units (RLUs) in transient transfections were determined by normalizing the CYP3A4-luc activity with the activity of the Renilla reporter.

In the cell cycle studies, unsynchronized or thymidine-synchronized HepG2 cells (clone 1; 20,000 cells per well, 100 µL per well) were seeded into 96-well tissue culture–treated solid white plates and treated with either DMSO or 10 µM rifampicin prior to luciferase assay. The cell number in each assay well was determined using alamar blue prior to luciferase assay, as described previously (30) with modifications. Briefly, 10 µL of alamar blue was added to each well 3 h prior to the measurement of signal. The fluorescence intensity of alamar blue was determined using an Envision plate reader (Perkin Elmer) with Ex540 and Em590 filters. Luminescence signal from the luciferase reporter was subsequently determined from the same plate. Luminescence signals from all luciferase assays were determined using an Envision plate reader. Normalized luciferase units (NLUs) in the cell cycle studies were determined by normalizing the CYP3A4-luc activity with the alamar blue activity.

**Screening for small molecules that activate the hPXR signaling pathway**- Clone 1 cells at 5,000 cells per well (25 µL per well) were seeded into 384-well tissue culture–treated solid white plates 24 h prior to compound treatment. Then, 25 nL of compound (10 mM in DMSO) was added to the
wells (final concentration was 10 µM) and the plates were incubated for 24 h before luciferase assay.

In total, 5,600 compounds representing drugs, drug candidates, known toxic compounds, and other molecules with characterized biological activities from Sigma (1,280 compounds), Microsource (Gaylordsville, CT; 3,200 compounds), and Prestwick (Illkirch, France; 1,120 compounds) were screened.

**PXR binding assay**- The TR-FRET hPXR competitive binding assay was performed accordingly to the manufacturer’s instructions (Invitrogen) with minor modifications. Briefly, the binding assays were performed in 384-well low-volume (20 µL per well) solid black plates with 5 nM GST-hPXR ligand-binding domain (LBD), 40 nM of fluorescent-labeled hPXR agonist (Fluormore PXR Green, also referred to as a “tracer”), 5 nM of terbium-labeled anti-GST antibody, and test compound at a variety of concentrations. DMSO was used as a negative control. A potent hPXR agonist, SR-12813 was used as a positive control.

In the reaction mixture, GST-hPXR forms a complex with the terbium-labeled anti-GST antibody and the tracer. Excitation of terbium (the donor) using a 340 nm excitation filter results in energy transfer to the fluorophore of the tracer. This energy transfer is detected by an increase in the fluorescence emission of the tracer at 520 nm and a decrease in the fluorescence emission of terbium at 495 nm. The FRET ratio was calculated by dividing the emission signal at 520 nm by the emission signal at 495 nm. A competitor compound such as SR-12813 replaces the tracer from the complex and decreases the FRET ratio accordingly. The reactions were incubated at 25°C for 30 min before measuring the fluorescent emission of each well at 495 nm and 520 nm using a 340 nm excitation filter, 100 µs delay time, and 200 µs integration time, on a PHERAStar plate reader (BMG Labtech, Durham, MA). Equal amounts of lysates were loaded into each lane on an SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane prior to being analyzed using Western blotting. Western blotting was performed as described previously (31).

**Cell cycle analysis**- Clone 1 cells were plated in modified Eagle’s minimal essential medium supplemented with 10% FBS, 2 mM L-glutamine, and 1% pen/strep. To synchronize the cells at the beginning of the S phase, we followed methodology using a high concentration of thymidine as previously reported by others (32,33) with minor modifications. Two synchronization protocols were used and similar results were obtained.

In the first protocol, the cells were treated for 48 h with 2 mM of thymidine. In the second protocol, the cells were treated for 24 h with 2 mM thymidine, released for 6 h by washing out the thymidine, and then blocked again with 2 mM thymidine for 48 h. The cells were then washed with PBS, trypsinized, and resuspended in culture medium (1x10⁶/mL), and either processed for fluorescence-activated cell sorter (FACS) analysis, or plated into assay plates and treated with either DMSO or 10 µM of rifampicin for 6 h prior to luciferase assays, or protein level determination. Cell cycle distribution was determined using flow cytometry. FACS analysis was performed at the time of harvesting for various treatments. FACS profiles are shown. Since the two blocking protocols produced similar results, we only present data generated using the first protocol in this report.

**RESULTS**

Inhibition of Cdk2 leads to activation of hPXR-mediated gene expression. Small-molecule
activators of the hPXR signaling pathway were sought using a cell-based screening assay based on the rationale that hPXR can be activated either by agonists, or by modulators of signaling pathways that cross-talk with the hPXR signaling pathway. A total of 5,600 compounds, including drugs, drug candidates, known toxic compounds, and other molecules with characterized biological activities, were tested. The screen was carried out in HepG2 human liver carcinoma cells stably transfected with hPXR (pcDNA3-hPXR) and CYP3A4-luc, in which the expression of luciferase was controlled by the hPXR-regulated CYP3A4 promoter. One of such stable clones, clone 1, was used in this study.

The HepG2 cell line has been commonly used to study hPXR regulation. While endogenous hPXR in HepG2 is undetectable using Western blotting, stable expression of hPXR from pcDNA3-hPXR in clone 1 was confirmed using Western blotting (data not shown). Clone 1 cells were plated into 384-well plates, incubated with compounds 24 h post-plating, and assayed for luciferase activity at 24 h after compound treatment. As shown for clone 1 in Figure 1A, rifampicin—an hPXR agonist used as a positive control in the screen—induces CYP3A4 promoter activation in a dose-dependent manner (with an EC_{50} of 1.3 µM). Two Cdk inhibitors—kenpaullone and roscovitine—strongly activated the CYP3A4 promoter, with an EC_{50} of 824 nM and 197 nM for kenpaullone and roscovitine, respectively (Figs. 1B & C and Table 1).

A compound can activate hPXR either through direct binding to hPXR (i.e., ligand-dependent hPXR activation) or through modulating a pathway which interacts with hPXR signaling (i.e., signal cross-talk), or involving both mechanisms. Compounds that involve both direct hPXR binding and signal cross-talk do exist. Forskolin is such a compound that not only binds to hPXR but also activates PKA, which in turn phosphorylates and potentiates hPXR activation (6). Forskolin was identified as a strong hPXR activator in our screen (data not shown).

To elucidate the mechanism of these Cdk inhibitors on activating hPXR, we first asked whether these compounds bind to hPXR. Figure 2 shows the various binding activities of SR-12813, kenpaullone, and roscovitine in a competitive binding assay (see Experimental Procedures for details). Table 1 compares the activity of these compounds in the cell-based reporter assay vs. in the binding assay. SR-12813, a potent hPXR agonist, strongly binds to hPXR LBD (IC_{50}=49 nM). The binding affinity of SR-12813 to hPXR in the binding assay is consistent with its activity in the reporter assay (EC_{50}=87 nM). While roscovitine binds to hPXR LBD with moderate affinity (IC_{50}=3,410 nM), kenpaullone is a much weaker hPXR binder (IC_{50}=39,270 nM). However, both kenpaullone and roscovitine activated hPXR in the cell-based reporter assay with moderate potency (EC_{50}=824 nM and 197 nM, respectively). While the ratio of IC_{50} (binding) to EC_{50} (cell-based) for SR-12813 is ~0.6, the ratio for kenpaullone and roscovitine is 47.7 and 17.3, respectively.

Therefore, Cdk inhibitors kenpaullone and roscovitine activate PXR with moderate potency, but bind to hPXR with lower affinity. These findings suggest that direct hPXR binding is not entirely responsible for the activation of hPXR by the Cdk inhibitors, and led us to hypothesize that inhibition of the Cdk pathway is involved in the regulation of hPXR activation.

Activation of Cdk2 attenuates hPXR-mediated gene expression. While kenpaullone and roscovitine are non-specific Cdk inhibitors, they are both potent Cdk2 inhibitors. Reported IC_{50} values for kenpaullone on Cdk2/cyclin E and Cdk2/cyclin A are 7,500 nM and 680 nM, respectively (34). Roscovitine inhibits both Cdk2/cyclin E and Cdk2/cyclin A with an IC_{50} of 700 nM (35). The potency of these compounds on inhibiting Cdk2 is consistent with their activity in the hPXR reporter assays (Table 1). Cdk2 has been shown to regulate NRs. If kenpaullone and roscovitine activate hPXR by inhibiting Cdk2, then activation of Cdk2 will lead to the attenuation of hPXR activation.

The activity of Cdk2 is regulated by two cyclins: cyclin A and cyclin E. We first tested the effect of Cdk2/cyclin E on the activity of hPXR. As shown in Figure 3A, transient co-expression of cyclin E, together with either a wild-type Cdk2 (Cdk2-WT), or a mutationally-activated Cdk2 (Cdk2-AF) that carries Thr^{14} to Ala^{14} and Tyr^{15} to Phe^{15} mutations and is unable to undergo inhibitory phosphorylation (13), down-regulated both basal (DMSO vehicle control) and rifampicin-induced (10 µM rifampicin) activation of hPXR in HepG2 cells. Induction of CYP3A4-luc was used to measure the activity of hPXR in this assay.

In contrast, either cyclin E alone, or co-expression of cyclin E with a catalytically inactive kinase-dead Cdk2 (Cdk2-KD) only marginally reduced the activity of hPXR. This marginal inhibition is probably owing to the effect of an increased cyclin E level on endogenous Cdk2.
Western-blotting analysis indicated that the expression levels of hPXR were not affected by co-expressions of different Cdk2 and cyclin E constructs (Fig. 3B), suggesting that the reduction in hPXR transactivity is due to the inhibitory effect of Cdk2 on hPXR, rather than a decrease in hPXR expression levels. Similar results were obtained when Cdk2/cyclin A was tested (Figs. 3C & D). Therefore, activation of Cdk2 leads to the attenuation of hPXR activation, while inhibition of Cdk2 causes activation of hPXR. These results indicate that Cdk2 negatively regulates hPXR activity.

Cdk2 phosphorylates hPXR. Phosphorylations of proteins by protein kinases have been shown to alter the function of the proteins. One of the possible mechanisms for Cdk2 to negatively regulate hPXR is by directly phosphorylating hPXR. Cdk2 often recognize and phosphorylate a Ser or a Thr, followed by a Pro (13). There are two such Ser or Thr residues in hPXR (Ser<sup>350</sup>-Pro<sup>351</sup> and Thr<sup>422</sup>-Pro<sup>423</sup>), suggesting that hPXR could be a substrate for Cdk2.

We therefore sought to determine whether Cdk2 could phosphorylate the hPXR protein. In an in vitro kinase assay, we demonstrated that reconstituted complexes of purified Cdk2 with either cyclin A or cyclin E directly phosphorylate purified hPXR (Fig. 4A & B). Histone H1, a known Cdk2 substrate with multiple phosphorylation sites, was used as a positive substrate control in the assays. Purified GST protein, which has been shown not to be a Cdk2 substrate by others (13), was used as a negative substrate control in the assays. Similar amounts of histone H1, hPXR, and GST were used in the kinase assays (Figs. 4C & D).

Histone H1 appeared to be a more efficient substrate of Cdk2, suggesting that there might be fewer Cdk2 phosphorylation sites in hPXR than in histone H1. These results indicate that Cdk2 directly phosphorylates hPXR in vitro.

A phosphorylation-deficient mutation at Ser<sup>350</sup> confers hPXR resistance to Cdk2. To provide further evidence that hPXR is the target of Cdk2, we decided to mutate a putative Cdk phosphorylation site and examine the functional consequences of the mutations. Ser<sup>350</sup> appears to be a preferred Cdk phosphorylation site, since it exists in a sequence (S<sup>350</sup>-P<sup>351</sup>-D<sup>352</sup>-R<sup>353</sup>, where S is Ser, P is Pro, D is aspartate, and R is arginine) that perfectly matches the consensus Cdk phosphorylation motif (S/T-P-X-R/K, where T is Thr, X is a polar amino acid such as D, and K is lysine) (36). If Ser<sup>350</sup> is a Cdk2 phosphorylation site, then mutation of Ser to a negatively-charged aspartate (hPXR<sub>350D</sub>), to mimic phosphorylation, will lead to attenuation of hPXR activity. In contrast, mutation of Ser to alanine (hPXR<sub>350A</sub>), which places a hydrophobic side chain at position 350 and renders hPXR<sub>350A</sub> deficient of phosphorylation, will confer resistance to the inhibitory effect of Cdk2.

Indeed, as shown in Figure 5A, while hPXR<sub>350A</sub> is indistinguishable from the wild-type hPXR in both basal and rifampicin-induced activation in HepG2 cells (lanes 1 and 2), the activity of hPXR<sub>350D</sub> is significantly reduced (lanes 1 and 3), suggesting that the phosphomimetic mutation of Ser<sup>350</sup> mimics the inhibitory effect of Cdk2.

To examine the sensitivity of the mutants to Cdk2, we co-transfected either wild-type or mutated hPXR with both cyclin E and Cdk2. Consistent with the observation shown in Figure 3A, Cdk2 down-regulated the activity of wild-type hPXR (Figure 5A, lanes 1 and 4). Interestingly, hPXR<sub>350A</sub> is less sensitive to the inhibitory effect of Cdk2 when compared to the wild-type hPXR (lanes 4 and 5), indicating that hPXR<sub>350A</sub> is partially phosphorylation-deficient for Cdk2, and suggesting that Ser<sup>350</sup> is a possible Cdk2 phosphorylation site. However, hPXR<sub>350A</sub> was not totally resistant to Cdk2 (lanes 2 and 5), suggesting that Ser<sup>350</sup> is not the only Cdk2 phosphorylation site. We also noticed that Cdk2 further reduced the activity of hPXR<sub>350D</sub> (lanes 3 and 6), again, supporting the notion that Ser<sup>350</sup> is not the only Cdk2 phosphorylation site.

hPXR<sub>350D</sub> was more sensitive to Cdk2 than the wild-type hPXR (lanes 4 and 6), probably because the transiently transfected Cdk2 only phosphorylated a portion of wild-type hPXR at Ser<sup>350</sup>. In contrast, all the Ser<sup>350</sup> residues of hPXR<sub>350D</sub> have been mutated to aspartate to mimic phosphorylation.

These results, together with the results shown in Figure 4, suggest that hPXR is the target of Cdk2, and phosphorylation of hPXR is indeed the point of regulation by Cdk2.

Western-blotting analysis confirmed that the impaired function of hPXR<sub>350D</sub> was not due to a reduction in protein expression, and that the resistance of hPXR<sub>350A</sub> to the inhibitory effect of Cdk2 was not due to an enhancement of protein expression (Fig. 5B).

hPXR-mediated gene expression is differentially regulated during the cell cycle in HepG2 cells. Cdk2 is a key regulator of cell cycle progression. The activity of Cdk2 fluctuates during the cell cycle, with activity peaks at both the G1/S checkpoint and during the S phase. Since
Cdk2 negatively regulates hPXR activity in HepG2 cells, we asked whether the activity of hPXR also changes during the cell cycle. To address this question, clone 1 was treated with 2 mM of thymidine for 48 h to synchronize the cells at the S phase of the cell cycle. Thymidine at 2 mM has been shown by others to arrest cells at the beginning of the S phase (32,33). While 65% of the unsynchronized cells were in G1 phase, thymidine treatment resulted in an enrichment of cells in the S phase (68%). The cell cycle distribution profiles were confirmed using FACS analysis (Fig. 6A). Both synchronized and unsynchronized cells were treated with either 10 μM of rifampicin or DMSO vehicle control for 6 h, and CYP3A4-luc activity was measured and normalized to total cell number, as explained in Experimental Procedures.

As shown in Figure 6B, CYP3A4 promoter activity, both basal and rifampicin-induced, was significantly reduced in thymidine-treated cells, when compared to the activity detected in the unsynchronized cells (i.e., ~60% and ~80% reduction for basal and rifampicin-induced CYP3A4 promoter activity). Western-blotting analysis of hPXR expression—using an antibody that has been shown to specifically recognize hPXR (37,38)—revealed similar levels in unsynchronized and synchronized clone 1 cells (Fig. 6C), indicating that the reduced hPXR activity (by measuring the CYP3A4 promoter activity) in S phase is not due to reduced expression of hPXR. In addition, we found that thymidine does not compete with hPXR agonist for binding to hPXR (data not shown), indicating that the reduced hPXR activity in S phase is not due to an antagonistic effect of thymidine.

Taken together, these results suggest that hPXR activity is differentially regulated during the cell cycle, with a significantly reduced activity in the S phase. This observation is consistent with our finding that hPXR activity is negatively regulated by Cdk2, whose activity peaks at the G1/S checkpoint as well as at the S phase.

**DISCUSSION**

hPXR and CYP3A4 are primarily expressed in liver and intestine. Although hepatocytes in normal adult liver are quiescent (G0 phase), hepatocytes passing through the cell cycle exist in a number of important physiological or diseased conditions, including liver development and liver regeneration (17-19,21). Significant changes in the expression of enzymes involved in drug metabolism occur during ontogeny, with a very low level of CYP3A4 in fetal liver (22,23). Levels of CYP3A4 expression are also reduced in regenerating livers in patients (20). In addition, extrahepatic expression of hPXR has been reported in other actively dividing cells such as cells from prostate cancer, endometrial cancers, and osteosarcoma, and the expression of hPXR in these tumors has been linked to the sensitivity of cells to therapeutic drugs (24-26).

We therefore speculated that the activity of hPXR would be regulated during the cell cycle. Since multiple cellular signaling pathways have been implicated in the modulation of activities of NRs, including hPXR, we designed an unbiased cell-based screening approach that would identify compounds that activate the hPXR signaling pathway. We screened a library of known bioactive compounds, and identified two Cdk inhibitors as potent hPXR activators.

We show in this report, for the first time, that the activity of hPXR is negatively regulated by Cdk2 (Figs. 1, 2 & 3), and that the activity of hPXR changes during the cell cycle, with significantly reduced activity observed in the S phase of the cell cycle (Fig. 6). The reduced hPXR activity observed in the S phase might be an underestimation, since thymidine treatment only increases the percentage of cells in S phase to 68% but not to 100% (Fig. 6). We also show that the differential hPXR activity observed during the cell cycle is not due to differential hPXR expression levels (Fig. 6).

Since reduction in drug metabolism enzymes, including CYP3A4, might have a profound effect on therapeutic efficacy and the risk of adverse drug reactions in the fetus and child, as well as adult patients with regenerating liver due to various liver injuries, understanding the molecular mechanisms responsible for these reductions is important for designing an effective therapy that prevents adverse drug interactions. Our finding that Cdk2 negatively regulates hPXR activity and CYP3A4 expression contributes to the understanding of such molecular mechanisms.

Other studies have shown that the activity of AR, PR, and glucocorticoid receptor (GR) is regulated during the cell cycle. While the activity of AR was shown to be higher in G0 and S phases, but lower in the G1/S transition (39), PR activity was found to be significantly higher in the S phase, but lower in the G1 and G2/M phases of the cell cycle (2). Our results suggest that the regulation of hPXR during the cell cycle is different from that of PR and AR. In addition, in contrast to PR, whose activity requires Cdk2
activity (15), hPXR activity is negatively regulated by Cdk2.

Our finding that hPXR activity is negatively regulated by Cdk2 is consistent with multiple reported results that show that hPXR activity and CYP3A4 expression are negatively regulated by growth factors in liver cells passing through the cell cycle (1). For example, Thasler et al. reported that during liver regeneration, augmenter of liver regeneration (ALR), a hepatotrophic factor, repressed both basal and rifampicin-induced CYP3A4 expression without affecting the expression of hPXR (40). In another study, hepatocyte growth factor (HGF) was shown to induce human hepatocyte proliferation and DNA synthesis, and repress both basal and rifampicin-induced CYP3A4 expression, as well as other CYPs. Interestingly, time-course studies suggested that the reduction in CYP expression occurs when DNA synthesis peaks (41).

We also report that Cdk2 directly phosphorylates hPXR in vitro (Fig. 4). In addition, we showed that a phosphomimetic mutation of Ser350, a putative Cdk phosphorylation site existing in a consensus Cdk phosphorylation motif (36), impairs the function of hPXR, while a phosphorylation-deficient mutation of Ser350 confers resistance to the inhibitory effect of Cdk2 (Fig. 5A). These results strongly suggest that phosphorylation of hPXR is indeed the point of regulation by Cdk2, and that hPXR is the target of Cdk2. However, it is highly possible that multiple Cdk2 phosphorylation sites exist in hPXR, since neither the phosphomimetic mutation nor the phosphorylation-deficient mutation of Ser350 render 100% resistance to the inhibitory effect of Cdk2. Extensive identification of all the Cdk2-regulated phosphorylation sites in hPXR will lead to more exciting discoveries regarding the regulation of hPXR by Cdk2-mediated phosphorylations.

Many xenobiots, including those examined in one of our previous studies (30), have been shown to transactivate hPXR in cell-based assays. Whether all of these hPXR transactivators function solely through direct binding to hPXR was unclear. Our current studies indicate that modulation of signaling pathways, such as the Cdk pathway, also contributes to the regulation of hPXR activity; supporting the hypothesis that hPXR functions not only as a xenobiotic receptor for direct binding of xenobiots, but also as a site of integration of various signaling pathways that interact with the hPXR signaling pathway. Our finding expands the roles of PXR as a “xenobiotic sensor.”

Intervention of signal transduction provides targeted therapeutic approaches for many diseases. Inhibitors of signaling pathways, including the Cdk pathway, are among such targeted therapies. Since hPXR has been found to express in multiple human tumors, and activation of hPXR in these tumors might lead to decreased efficacy of therapeutic drugs, our finding that Cdk2 negatively regulates hPXR might have important implications in the design of effective therapies. For example, we would anticipate that therapeutic drugs that inhibit Cdk2 will lead to activation of hPXR, which might lead to decreased efficacy of therapeutic drugs.

Our finding that hPXR is regulated by Cdk2 reveals a potential mechanism for the differential regulation of CYP3A4 expression in hepatocytes during the cell cycle, and also highlights the importance to include the consideration of cell cycle status when analyzing the activity of PXR and expression of CYPs.

REFERENCES

FOOTNOTES
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The abbreviations used are: DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; pen/strep, penicillin/streptomycin; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TR-FRET, time-resolved fluorescence resonance transfer.

**FIGURE LEGENDS**

**Fig. 1.** Inhibition of Cdk2 leads to activation of the hPXR-mediated gene expression. A. HepG2 stably transfected with hPXR and CYP3A4-luc (clone 1) responds to rifampicin in a dose-responsive manner. Cells were treated for 24 h with indicated concentrations of rifampicin prior to luciferase assay. B & C. Kenpaullone (B) and roscovitine (C) activate CYP3A4-luc in clone 1. Cells were treated for 24 h with indicated concentrations of compounds prior to luciferase assay. CYP3A4 promoter activity is expressed as a percentage of activation (% Act) by normalizing with luciferase activity from 10 µM of rifampicin, an internal control included in each assay plate (see Experimental Procedures for details). Compounds were tested at 10 concentrations in triplicate in three different plates.

**Fig. 2.** Kenpaullone and roscovitine are weak hPXR binders in a TR-FRET hPXR competitive binding assay. The assays were performed as described in Experimental Procedures. A potent hPXR agonist SR-12813 was used as a positive control. The FRET ratio was calculated by dividing the emission signal at 520 nm (emission from acceptor fluorophore) by the emission at 495 nm (emission from donor terbium). Binding of SR-12813 to hPXR decreased the FRET ratio. Compounds were tested at 15 concentrations in triplicate.

**Fig. 3.** Activation of Cdk2 leads to attenuation of hPXR-mediated gene expression. A & C. Cdk2 inhibits hPXR transactivation. HepG2 cells were co-tranfected with FLAG-hPXR, CYP3A4-luc, other plasmids as indicated (V5-cyclin E, V5-Cdk2, or cyclin A) and CMV-Renilla luciferase plasmid (as a transfection control). Cells were treated with DMSO or 10 µM of rifampicin 24 h post-transfection. Luciferase activities were measured 24 h after compound treatments. The relative luciferase units (RLUs) were determined by normalizing with the Renilla luciferase control. The values represent the means of six independent experiments, and the bars denote the standard deviation. The p value was ascertained using the Student’s t test and expressed as ***p < 0.001, *p < 0.05, and ns (not significant; p > 0.05). Comparisons were made with samples that were not transfected with either cyclin E or Cdk2 (the leftmost lane in A or C), for either DMSO or rifampicin-treated samples, respectively. B & D. Expression of hPXR is not affected by co-expression of Cdk2 and/or cyclin E/A. Actin expression level is used to verify equal loading of lysates. α-FLAG: anti-FLAG; α-V5: anti-V5; α-β-actin: anti-β-actin. Cdk2-WT, wild-type V5-Cdk2; Cdk2-AF, activated V5-Cdk2; Cdk2-KD, inactive V5-Cdk2. Data shown is from a representative experiment.

**Fig. 4.** Cdk2 phosphorylates hPXR in vitro. Reconstituted Cdk2/cyclin A (20 ng, A) or Cdk2/cyclin E (20 ng, B) were used with 1 µg of substrate, as indicated. The kinase assays were performed as described in Experimental Procedures. The amounts of input substrate are shown in C (for Cdk2/cyclin A) and D (for Cdk2/cyclin E), as revealed through SimplyBlue staining. H1, histone H1; M, molecular weight marker.

**Fig. 5.** Mutagenesis analysis of Ser^{350}. A phosphorylation-deficient mutation at Ser^{350} confers hPXR resistance to Cdk2. HepG2 cells were co-transfected with CYP3A4-luc, other plasmids as indicated (cyclin E, Cdk2, or hPXR), and CMV-Renilla luciferase plasmid (as a transfection control). Cells were treated with DMSO or 10 µM of rifampicin 24 h post-transfection. Luciferase activities were measured 24 h after compound treatments. The relative luciferase units (RLUs) were determined by normalizing with the Renilla luciferase control. The values represent the means of six independent experiments, and the bars denote the standard deviation. In the absence of cyclin E and Cdk2 transfection (lanes 1, 2, and 3; lane 1 is the leftmost lane), the p value was ascertained using the Student’s t test and expressed as ***p <
0.001, and ns (not significant; p > 0.05), as compared with samples that were not transfected with either cyclin E or Cdk2 (lane 1), for either DMSO or rifampicin-treated samples, respectively. In the presence of cyclin E and Cdk2 transfection (lanes 4, 5, and 6; lane 6 is the right-most lane) the p value was ascertained using the Student’s t test and expressed as ###p < 0.001, as compared with samples that were not transfected with either cyclin E or Cdk2 (lane 4 versus lane 1, lane 5 versus lane 2, and lane 6 versus lane 3), for either DMSO or rifampicin-treated samples, respectively. Statistical significant differences between other samples (noted in brackets) are indicated by p < 0.001. B. Expression levels of hPXR. Actin expression level is used to verify equal loading of lysates. α-FLAG: anti-FLAG; α-β-actin: anti-β-actin. Cdk2-WT, wild-type V5-Cdk2; cyclin E, V5-cyclin E. Data shown is from a representative experiment.

**Fig. 6.** CYP3A4 promoter activity is significantly reduced in the S phase of the cell cycle. A. FACS analyses obtained from the unsynchronized and synchronized HepG2 cells (clone 1). B. CYP3A4 promoter activity is reduced in the S phase of the cell cycle. Synchronized (Syn) or unsynchronized (Unsyn) cells were treated with DMSO or 10 µM of rifampicin for 6 h prior to luciferase assays. Normalized luciferase units (NLU) were determined by normalizing with total cell number as determined using alamar blue, as described in Experimental Procedures. NLU is shown at the top of the bar for each sample. Percentages of cells in G1, S, and G2/M are indicated. The values represent the means of four independent experiments and the bars denote the standard deviation. The p value was ascertained using the Student’s t test and expressed as ***p < 0.001, as compared with unsynchronized samples, for either DMSO or rifampicin-treated samples, respectively. C. hPXR levels were not changed as a result of thymidine treatment. hPXR levels in unsynchronized or synchronized clone 1 cells were determined using Western blotting. Data shown is from a representative experiment.
Table 1. Comparison of compound activities from various assays.

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC$_{50}$ (hPXR reporter assay)</th>
<th>IC$_{50}$ (hPXR binding assay)</th>
<th>IC$_{50}$ (Cdk inhibition) (34,35)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cdk2/cyclin E</td>
</tr>
<tr>
<td>SR-12813</td>
<td>87 nM</td>
<td>49 nM</td>
<td>N/T</td>
</tr>
<tr>
<td>Roscovitine</td>
<td>197 nM</td>
<td>3,410 nM</td>
<td>700 nM</td>
</tr>
<tr>
<td>Kenpaullone</td>
<td>824 nM</td>
<td>39,270 nM</td>
<td>7,500 nM</td>
</tr>
</tbody>
</table>

N/T: not tested
Figure 1

A

B

C

Log[Rifampicin] (M)

Log[Kenpaullone] (M)

Log[Roscovitine] (M)

CYP3A4 promoter activity (% Act)
Figure 2

![Graph showing the relationship between log[Test Compound] (M) and 520 nm/495 nm ratio for SR12813, Roscovitine, and Kenpaullone.](image)
Figure 4

A  No substrate  H1  GST  hPXR

50kD →

B

$^{32}$P

C  M  No substrate  H1  GST  hPXR

Cdk2/cyclin A

D  Substrate inputs

Cdk2/cyclin E
Figure 5

A

B
Figure 6

A

Unsynchronized

Number of cells

Synchronized

Number of cells

DNA Content

DNA Content

B

CYP3A4 promoter activity (NLD)

62.3

10.4

15.0

DMSO

Rifampicin

Unsyn

Syn

Unsyn

Syn

hPXR

β-actin

α-hPXR

α-β-actin

G1 (%)

S (%)

G2/M (%)

17

68

15

15
Cyclin-dependent kinase 2 negatively regulates human pregnane X receptor-mediated CYP3A4 gene expression in HepG2 liver carcinoma cells

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