The Pregnane X Receptor: A Promiscuous Xenobiotic Receptor That Has Diverged during Evolution


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Transcription of genes encoding cytochrome P450 3A (CYP3A) mono-oxygenases is induced by a variety of xenobiotics and natural steroids. There are marked differences in the compounds that induce CYP3A gene expression between species. Recently, the mouse and human pregnane X receptor (PXR) were shown to be activated by compounds that induce CYP3A expression. However, most studies of CYP3A regulation have been performed using rabbit and rat hepatocytes. Here, we report the cloning and characterization of PXR from these two species. PXR is remarkably divergent between species, with the rabbit, rat, and human receptors sharing only approximately 80% amino acid identity in their ligand-binding domains. This sequence divergence is reflected by marked pharmacological differences in PXR activation profiles. For example, the macrolide antibiotic rifampicin, the antidiabetic drug troglitazone, and the hypocholesterolemic drug SR12813 are efficacious activators of the human and rabbit PXR but have little activity on the rat and mouse PXR. Conversely, pregnane 16α-carbonitrile is a more potent activator of the rat and mouse PXR than the human and rabbit receptor. The activities of xenobiotics in PXR activation assays correlate well with their ability to induce CYP3A expression in primary hepatocytes. Through the use of a novel scintillation proximity binding assay, we demonstrate that many of the compounds that induce CYP3A expression bind directly to human PXR. These data establish PXR as a promiscuous xenobiotic receptor that has diverged during evolution. (Molecular Endocrinology 14: 27–39, 2000)

INTRODUCTION

The liver and intestine are important sites for the metabolism of both endogenous and exogenous chemicals. Members of the cytochrome P450 (CYP) superfamily of hemoproteins play critical roles in the oxidative metabolism of compounds in both of these tissues. The CYP3A gene products are among the most abundant of the mono-oxygenases in mammalian liver and intestine. In humans, CYP3A4 is involved in the metabolism of more than 50% of all drugs as well as a variety of other xenobiotics and endogenous substances, including steroids (1).

Expression of CYP3A genes is induced by a variety of compounds, including many drugs (1, 2). The induction of CYP3A transcription represents the basis for a number of common drug-drug interactions. Many xenobiotics have been profiled for their effects on CYP3A expression in primary hepatocytes from rabbits or rats (3–5). These studies have revealed marked species differences and called into question the validity of using animal models or nonhuman hepatocytes for predicting the effects of xenobiotics on CYP3A transcription in humans. Transfection studies in which reporter genes driven by CYP3A promoter sequences were introduced into rabbit or rat hepatocytes showed these differences were a consequence of host cell factors rather than differences in cis-acting sequences in the CYP3A gene promoters (5). However, the mo-
Molecular basis for these species differences had remained in question.

We and others recently cloned novel mouse and human orphan members of the nuclear hormone receptor superfamily and showed that they are activated by a variety of known inducers of CYP3A expression (6–10). We have named these orphan nuclear receptors pregnane X receptors (PXRs) based upon their efficacious activation by natural C21 steroids (pregnanes). Mouse and human PXR are predominantly expressed in liver and intestine and bind to xenobiotic response elements previously identified in the human and rat CYP3A promoters (6–9). Based upon these data, PXR has been suggested to serve as a key regulator of CYP3A expression. The human and mouse PXR share only 76% amino acid identity in their ligand-binding domains (LBDs) and display markedly different activation profiles in response to xenobiotics. Thus, it has remained an open question whether these receptors are bona fide orthologs or members of a broader subfamily of closely related orphan nuclear receptors.

We now report the cloning and characterization of PXR from rabbit and rat, two species that are frequently used for studies of drug metabolism and CYP3A regulation. Although PXR has diverged significantly during the course of evolution, our results provide evidence that it has an important role in CYP3A regulation in multiple species. In addition, we also report the development of a scintillation proximity binding assay for human PXR and show for the first time that structurally diverse compounds bind directly to this orphan nuclear receptor.

RESULTS

Cloning and Characterization of the Rabbit and Rat PXR

Because pharmacological and toxicological studies are often conducted in rabbits and rats, we sought to clone PXR from these species. PCR strategies were employed using oligonucleotides derived from the mouse and human PXR and cDNA prepared from either rat or rabbit liver. The resulting rat and rabbit PXR clones encode proteins of 431 and 411 amino acids, respectively (Fig. 1A). Rat PXR is closely related to its mouse ortholog, sharing 97% amino acid identity throughout. Alignment of the PXR sequences revealed interesting differences between species. Although their DNA-binding domains (DBDs) are approximately 95% identical, the LBDs of the rabbit, rodent, and human PXR share only about 80% amino acid identity (Fig. 1). Notably, the rabbit PXR is roughly equally divergent from the human and rodent PXR (Fig. 1B). This degree of divergence is unprecedented for nuclear receptor orthologs. Secondary structure prediction within the LBD suggested that several of the residues that differ between species might affect the ligand-binding properties of the receptor (Fig. 1A). This analysis also revealed the presence of a large insert between the predicted H2 and H3, similar to that observed in the peroxisome proliferator-activated receptors (PPARs) but not the classical steroid receptors (11–13).

The tissue expression patterns of rat and rabbit PXR were determined by Northern analysis using blots containing poly(A)+ RNA from a variety of adult tissues. Rat and rabbit PXR are most abundantly expressed in the liver, with PXR mRNA also detected in tissues of the gastrointestinal tract (Fig. 2). Two distinct transcripts of approximately 2.5 and 4.0 kb were seen in rat liver. The 2.5-kb transcript is also observed in rat stomach and small intestine. PXR transcripts of 2.6, 4.5, and 5.0 kb were detected in rabbit liver, small intestine, and kidney. We did not detect PXR in rat kidney, even in longer exposures of the blot. This was surprising since PXR was previously observed in mouse kidney (6). We conclude that PXR is most abundantly expressed in liver in all four species, but that differences exist in PXR transcript size and extrahepatic tissue expression.

Differential Activation of Mouse, Rat, Rabbit, and Human PXR

A variety of different xenobiotics were previously tested for their ability to induce CYP3A expression in primary hepatocytes from either rabbits or rats (3–5). Many of these compounds, including dexamethasone, phenobarbital, RU486, spironolactone, clotrimazole, and trans-nonachlor, induced CYP3A expression in hepatocytes from both species (4). However, there were notable differences. For example, the macrolide antibiotic rifampicin was a much more efficacious inducer of CYP3A expression in rabbit hepatocytes than rat hepatocytes. Conversely, pregnenolone 16a-carbonitrile (PCN) induced CYP3A expression in rat hepatocytes but not rabbit hepatocytes. We tested this same panel of compounds for activation of the rabbit and rat PXR. PXR expression plasmids were cotransfected into CV-1 cells together with a reporter plasmid containing two copies of the CYP3A1 direct repeat 3 (DR-3) PXR response element upstream of the minimal thymidine kinase promoter and chloramphenicol acetyltransferase (CAT) gene. The cells were then treated with 10 μM concentrations of each compound except for phenobarbital, which was tested at 1 mM. Both the rabbit and rat PXR responded to xenobiotics. Rifampicin and dexamethasone were the most efficacious activators of rabbit PXR, inducing reporter levels more than 15-fold over the basal level (Fig. 3A). The synthetic steroids PCN, RU486, cyproterone acetate (CPA), and spironolactone were efficacious activators of rat PXR, increasing reporter levels more than 7-fold (Fig. 3A). In general, activation of the rat and rabbit PXR agreed with the reported induction of CYP3A expression in primary hepatocytes from these same species (4). Rifampicin was an efficacious activator of...
Fig. 1. Sequence Comparison of PXR among Species

A, Alignment of the rabbit, rat, human, and mouse PXR amino acid sequences. Residues that differ from the consensus are boxed in black. The DBD and the predicted α-helices in the LBD are indicated. B, The percent amino acid identities and differences are indicated for the PXR DBD and LBD. 1, Rabbit; 2, rat; 3, mouse; 4, human.
rabbit PXR but had no effect on rat PXR (Fig. 3A). Although both receptors were activated by 10 μM concentrations of PCN, full dose-response analysis revealed that PCN was roughly 1 order of magnitude more potent on rat PXR than rabbit PXR (Fig. 3B). These data suggest that PXR has an important role in the regulation of CYP3A expression in multiple species.

The same panel of CYP3A inducers was also tested on the human and mouse PXR (Fig. 3A). Based on the finding that rifampicin is a much more efficacious activator of CYP3A expression in hepatocytes from rabbits and humans than from rats (4, 5), together with the observation that rifampicin is an efficacious activator of human PXR (7–9), it had been suggested that the activation profiles of the rabbit and human PXR were likely to be similar. Indeed, the human and rabbit PXR were both efficiently activated by rifampicin as well as by RU486, clotrimazole, trans-nonachlor, and phenobarbital (Fig. 3A). However, the rabbit PXR was much more sensitive than its human ortholog to activation by the synthetic steroids dexamethasone, PCN, CPA, and spironolactone (Fig. 3A). Thus, there are clear differences in responsiveness of rabbit and human PXR to xenobiotics. Given their high degree of sequence identity, it was not surprising that the rat and mouse PXR had very similar activation profiles, although there were subtle differences (Fig. 3A). We conclude from these studies that although the human, rabbit, rat, and mouse PXR are activated by several of the same compounds, each is pharmacologically distinct.

Among the established inducers of CYP3A expression that we tested for PXR activation was troglitazone. Troglitazone is a member of the thiazolidinedione class of insulin-sensitizing drugs that lower glucose, lipid, and insulin levels in patients with type 2 diabetes. Thiazolidinediones mediate their therapeutic effects by binding and activating the nuclear receptor PPARγ (14). Troglitazone is known to increase CYP3A4 activity and to enhance the metabolism of other drugs in patients (15). Consistent with this, we found that troglitazone activated both the human and rabbit PXR (Fig. 3A). Full dose-response analysis showed that troglitazone activated human PXR with an EC₅₀ value of approximately 3 μM, which is comparable to the concentration required to activate PPARγ (data not shown). Thus, the interaction of troglitazone with other drugs is likely to result from its activation of PXR. Interestingly, troglitazone had little effect on the rat and mouse PXR.

**Rexinoids Activate PXR**

Like many of the other orphan nuclear receptors, PXR binds to its hormone response elements as a heterodimer with retinoid X receptor (RXR) (6–9). These heterodimers have been classified as either permissive or nonpermissive depending on whether they are activated by RXR ligands (rexinoids) (16). To test whether the PXR/RXR heterodimer is permissive for activation by rexinoids, we performed cotransfection assays with PXR from the four species in CV-1 cells in the presence of the natural RXR ligand 9-cis-retinoic acid and the synthetic, RXR-selective compounds LGD1069 and LG100268 (17, 18). The RXR ligands did not activate the PXR/RXR heterodimer at the nanomolar concentrations that are typically required to activate the RXR homodimer or the permissive RXR heterodimers. However, micromolar concentrations of the rexinoids did weakly activate the heterodimers formed between either the human or rabbit PXR and RXR (Fig. 4). Transfection experiments performed with saturating concentrations of a PXR ligand showed that LG100268 did not further activate the human PXR/RXR heterodimer (data not shown). Notably, the rexinoids had no effect on the rat or mouse PXR heterodimers with RXR (data not shown), suggesting that these compounds might not mediate their effects through RXR but rather via the rabbit and human PXR. Consistent with this idea, we have shown that RXR ligands bind directly to human PXR at micromolar concentrations (see below). Thus, our data indicate that the heterodimers formed between either the human or rabbit PXR and RXR can be activated by micromolar concentrations of rexinoids that cross-react with PXR.

**SR12813 Is a Potent PXR Activator**

The bisphosphonate ester SR12813 lowers cholesterol levels in a range of species including rats, dogs, and primates (19, 20). The molecular mechanism for these hypocholesterolemic effects has remained unclear. Since SR12813 has been reported to increase CYP3A protein levels in rat hepatocytes (21), we...
tested its ability to activate PXR. SR12813 was a very potent and efficacious activator of both the human and rabbit PXR, with EC₅₀ values of approximately 200 nM and 700 nM, respectively (Fig. 5A). This is the most potent PXR activator to be identified to date. By contrast, SR12813 was only a very weak activator of the rat and mouse PXR (Fig. 5A).

We next tested whether the differences between rat, rabbit, and human PXR in response to SR12813 would be reflected at the level of CYP3A induction in primary hepatocytes derived from each of these species. Hepatocytes were treated with vehicle alone, SR12813, PCN, or rifampicin, and CYP3A mRNA levels were determined by Northern blot analysis using probes for CYP3A1, CYP3A4, and CYP3A6, major inducible CYP3A family members in rat, human, and rabbit, respectively. As expected, rifampicin was an efficacious inducer of CYP3A expression in human and rabbit hepatocytes, but not rat hepatocytes (Fig. 5B). Conversely, PCN induced CYP3A expression in rat but not human or rabbit hepatocytes. In agreement with the results from the transfection studies, SR12813 induced CYP3A expression in human and rabbit hepatocytes but only weakly in rat hepatocytes.
These results demonstrate that the PXR activation profile is predictive of the effects of SR12813 on CYP3A expression in primary hepatocytes from different species.

Structurally Diverse Xenobiotics Are PXR Ligands

The structural diversity of the compounds that activate PXR is unprecedented for a nuclear receptor. Do these xenobiotics, which range in mol wt from 232 (phenobarbital) to 823 (rifampicin), mediate their effects through direct interactions with PXR? We set out to address this issue by establishing a competition binding assay employing \[^{3}H\]SR12813 as a radioligand. Initial attempts to express the LBD of human PXR in *Escherichia coli* were unsuccessful due to its lack of solubility. However, coexpression of an 88-amino acid region of the steroid coactivator protein 1 (SRC-1) with the human PXR LBD resulted in soluble protein that was purified to homogeneity and biotinylated for use in ligand-binding assays. A scintillation proximity-binding assay was developed using streptavidin-coated polystyrene beads and the biotinylated human PXR. \[^{3}H\]SR12813 interacted specifically with human PXR with a dissociation constant (K_d) of 40 nM (Fig. 6A). This value is in good agreement with the EC_{50} value for SR12813 for activation of human PXR in the transfection assay (Fig. 5A). These data demonstrate that \[^{3}H\]SR12813 binds directly to human PXR.

We next tested various PXR activators for their ability to compete with \[^{3}H\]SR12813 for binding to human PXR. Each compound was tested at 10 \(\mu\)M except for phenobarbital, which was tested at the 1 mM concentration required to activate human PXR. Notably, all the xenobiotics that activated human PXR in the transfection assay displaced \[^{3}H\]SR12813 from the receptor (Fig. 6B). The compounds that interacted with human PXR included the thiazolidinedione trolitazone and the rexinoids 9-cis retinoic acid, LGD1069, and LG100268. Consistent with their relative inactivity in the transfection assay, the synthetic steroids PCN, CPA, spironolactone, and dexamethasone competed only weakly with \[^{3}H\]SR12813 for binding to human PXR (Fig. 6B). These data demonstrate that a variety of xenobiotics are capable of interacting with the PXR LBD at concentrations that are consistent with those required to activate the receptor in transfection assays. Given the high concentration of phenobarbital that is required for competition in the binding assay and the fact that it is known to mediate effects through other mechanisms (22), we cannot rule out the possibility that this barbiturate activates PXR through other signaling pathways.

Natural Steroids Are PXR Ligands

Human and mouse PXR are activated by a variety of naturally occurring steroids, among which C21 steroids (pregnanes) are the most potent (6–9). We tested various pregnanes and other steroids for their activities on the human, rabbit, rat, and mouse PXR in the transfection assay. Each PXR displayed a distinct activation profile (Fig. 7A). However, in each case, the most efficacious activator was a pregnane. 5\(\beta\)-Pregnan-3,20-dione was the most efficacious natural activator of the human, rat, and mouse PXR (Fig. 7A). Although 5\(\beta\)-pregnan-3,20-dione also activated the rabbit PXR, the closely related compound 17-OH progesterone was the most efficacious activator of the rabbit receptor. Weaker activation of PXR was also seen with other steroids, including corticosterone, dihydrotestosterone, and estradiol (Fig. 7A), as previously described (8, 9).

We examined whether these naturally occurring steroids interacted directly with human PXR. In agree-
ment with the transfection data, 5β-pregnane-3,20-dione competed most efficiently with [3H]SR12813 for binding to human PXR (Fig. 7B). Full dose-response analysis showed that 5β-pregnane-3,20-dione bound to human PXR with an IC_{50} value of approximately 400 nM (data not shown). Competition was also seen with 10 μM concentrations of other natural steroids that activate human PXR including corticosterone and estradiol (Fig. 7B). These data demonstrate that natural steroids can bind directly to human PXR and, furthermore, suggest that the natural PXR ligand is likely to be a metabolite or close analog of 5β-pregnane-3,20-dione.

DISCUSSION

Studies performed over the past 20 yr have revealed marked species differences in the induction of CYP3A expression in response to xenobiotics. These differences have complicated the development of assays to identify compounds that modulate CYP3A transcription. It was recently shown that the human and mouse orthologs of PXR are activated by compounds that induce CYP3A expression (6–9). We have now extended these earlier analyses to include rabbit and rat PXR. The characterization of PXR from these two species was of particular interest since a number of inducers of CYP3A expression have been studied in rabbit and rat hepatocytes, which are readily available. Overall, we find that there is a good correlation between PXR activation and the induction of CYP3A transcription in rat and rabbit hepatocytes. These data provide strong evidence that PXR is a key regulator of CYP3A transcription in a range of species, and that much of the cross-species variability in CYP3A regulation can be accounted for at the level of PXR activation. We note that several of the xenobiotics that induce CYP3A expression, including dexamethasone and phenobarbital, activate other nuclear receptors (22). Thus, other signaling pathways are also likely to contribute to the regulation of CYP3A expression.

Since rifampicin is an efficacious inducer of CYP3A expression in human and rabbit but not in rat, it was recently postulated that the rabbit PXR might be more closely related to its human ortholog than the rat PXR (9). In fact, the human, rabbit, and mouse/rat PXR are all roughly equally divergent, sharing only approximately 80% amino acid identity in their LBDs. Although both the rabbit and human PXR are activated by rifampicin, there are marked differences in their responsiveness to synthetic steroids such as dexamethasone and estradiol.
methasone and CPA. These differences in PXR activation profiles are likely to be reflected at the level of CYP3A expression in vivo. Thus, caution must be exercised in extrapolating CYP3A induction data for a particular compound from rabbit to man. Despite their divergence, several lines of evidence suggest that that PXRs are orthologs and not closely related members of a subfamily of nuclear receptors. First, each PXR is most abundantly expressed in the liver and tissues of the gastrointestinal tract. Second, our pharmacological data strongly suggest that each PXR regulates CYP3A expression in its respective species. Finally,

Fig. 6. SR12813 and Other Xenobiotics Bind to Human PXR
A. Purified human PXR LBD immobilized on SPA beads was incubated with concentrations of [3H]SR12813 ranging from 0.5 nM to 1000 nM in the absence (total binding, open squares) or presence (specific binding, closed triangles) of 10 μM clotrimazole to define nonspecific binding. Data points represent the mean of assays performed in triplicate. The Kd value for [3H]SR12813 binding to human PXR was 41 nM as calculated by nonlinear regression. B. Competition binding assays were performed with 10 nM [3H]SR12813 and 10 μM of each of the xenobiotics, except phenobarbital, which was tested at 1 mM. Data represent the mean of assays performed in duplicate ± SE and are plotted as percent inhibition of [3H]SR12813 binding where competition with unlabeled SR12813 is defined as 100%.
our searches of the expressed sequence tag (EST) databases have not revealed any other PXR-like sequences (J. T. Moore, unpublished results). The divergence in PXR could represent either an adaptive response to different environmental xenobiotic challenges or differences in natural PXR ligands be-

**Fig. 7. Natural Steroids Are PXR Ligands**

A, CV-1 cells were transfected with expression plasmids for PXR from the different species and the (CYP3A1 DR3)_2-tk-CAT reporter. Cells were treated with 10 μM of each steroid. Cell extracts were subsequently assayed for CAT activity. Data represent the mean of assays performed in triplicate ± SE. B, Competition binding assays were performed with purified human PXR LBD immobilized on SPA beads, 10 nM [³H]SR12813, and 10 μM of each steroid. Data represent the mean of assays performed in duplicate ± SE and are plotted as percent inhibition of [³H]SR12813 binding where competition with 10 μM unlabeled SR12813 is defined as 100%.
tween species. Although pregnanes are the most efficacious natural activators of PXR from each of the species, we have observed cross-species differences in PXR activation by natural steroids. However, the concentrations of these steroids required to activate PXR are superphysiological, and the natural ligand for PXR remains to be determined.

The known ligands for nuclear receptors are all small molecules with similar volumes and molecular weights (23). Larger molecules, such as growth factors, can also activate nuclear receptors through binding to cell surface receptors and activation of their second messenger-signaling cascades (24). The observation that rifampicin, a macrolide antibiotic with a mol wt of 823, activated human PXR and promoted its interaction with the coactivator SRC-1 in an in vitro coprecipitation assay was surprising (7–9). Could PXR bind to a ligand as large as rifampicin? The development of a radioligand competition binding assay for this orphan receptor has allowed us to address this question. Using [3H]SR12813 in a scintillation proximity assay, we have demonstrated that many of the xenobiotics, including rifampicin, that induce CYP3A4 expression bind directly to PXR. The interaction of rifampicin with PXR suggests that its ligand-binding pocket must be very large in comparison with other nuclear receptors. The only other nuclear receptors that are known to have such large ligand-binding pockets are the PPARs (11–13). X-Ray crystallography has established that the large cavities in the PPARs are due, in part, to the presence of an α-helix termed H2’ that is not present in other nuclear receptors. Sequence alignment suggests that PXR has an even larger insert in the H2’ region than the PPARs (Fig. 1A). Thus, it is possible that the promiscuity of PXR is due to the presence of a ligand-binding pocket that is larger than those of other nuclear receptors.

SR12813 lowers plasma cholesterol levels in a number of species including primates (19, 20). Although SR12813 has been reported to reduce cholesterol biosynthesis, by increasing the degradation of hydroxy-methylgluturate-coenzyme A reductase (20), the molecular target for its actions remains unknown. Despite its potency in activating human and rabbit PXR, we believe it unlikely that SR12813 mediates its hypcholesterolemic effects exclusively through PXR. PCN is a potent activator of the mouse and rat PXR and has effects on bile composition in rats (25). However, in agreement with previous studies (25), we have found that treatment of wild-type rats with PCN does not decrease serum cholesterol levels (J. L. Shenk and D. Winegar, unpublished data). Under these same conditions, SR12813, which is only a very weak activator of rat PXR, effectively lowers cholesterol levels. Moreover, rifampicin, which is widely used to treat tuberculosis at doses that induce CYP3A4 expression, has not been associated with reductions in cholesterol levels. These results suggest that PXR activation alone is unlikely to account for the hypcholesterolemic actions of SR12813. It was recently shown that micro-

molar concentrations of SR12813 activate the related nuclear receptor farnesoid X receptor (FXR) (26). FXR is a bile acid receptor that regulates genes such as cholesterol 7α-hydroxylase that are involved in cholesterol homeostasis (27–29). Thus, SR12813 may exert its hypcholesterolemic effects through FXR or other cellular receptors.

The thiazolidinedione antidiabetic drugs were developed using rodent models of insulin resistance, without knowledge of their cellular target. It is now known that these insulin sensitizers mediate their effects through activation of the PPARγ/RXR heterodimer (30, 31). Troglitazone is the first of these drugs to be marketed for the treatment of type 2 diabetes. Although the drug is devoid of serious side effects in rodents, in humans it has been shown to increase CYP3A4 activity (15) and is also associated with an idiosyncratic hepatotoxicity (32). Our data showing that troglitazone activates human PXR at concentrations similar to those required to activate PPARγ provides an explanation for its interactions with other drugs, including oral contraceptives. Interestingly, the relative lack of activity of troglitazone on the mouse or rat PXR may explain why these effects were not reported in animal toxicology studies. Additional studies will be required to determine whether PXR also plays a role in the hepatotoxicity observed with troglitazone. In this regard, it is interesting that the PXR ligand rifampicin has also been associated with hepatotoxicity in humans (33, 34). Our data suggest that certain rexinoids, which have been proposed as diabetes drugs (35), may show side effects similar to troglitazone through activation of PXR. The availability of PXR screening assays should allow for the development of new drugs for type 2 diabetes with increased selectivity for their cellular target, the PPARγ/RXR heterodimer.

In summary, we have demonstrated that PXR is a promiscuous nuclear xenobiotic receptor with an LBD that has diverged considerably during the course of evolution. This divergence in the PXR LBD accounts for the differential effects of various compounds on CYP3A expression across species. Comparative functional studies using rabbit and rat PXR will increase our ability to evaluate metabolism data from relevant nonhuman pharmacological model systems. Moreover, the availability of PXR in a high throughput binding format provides a valuable tool for the rapid identification of compounds that will induce CYP3A expression and interact with other drugs. Through the early elimination of these compounds from the drug discovery process, these assays will aid in the development of safer medicines.

MATERIALS AND METHODS

Reagents

ITS+ (insulin, transferrin, selenium, linoleic acid, and BSA supplement), rat tail collagen, type I, and Matrigel were pur-
Pregnane X Receptor 37

Structure was identified from the x-ray structure of the PPAR

The program MVP (37) was used to align the PXR sequences

rat PXR clone was reported by another group (36).

were se-

was 5

Inc.) and oligonucleotides derived from the 5

plification using rat liver cDNA (CLONTECH Laboratories, Inc. (Palo Alto, CA)). An internal 861-bp rabbit PXR fragment was first obtained by PCR using oligonucleotides derived from the mouse PXR LBD sequence (6). The forward oligonucleotide sequence was 5'-AAGTGGCTGGAGAGTTG-GCATG, and the reverse primer was 5'-TCCGAGTCGACT-GAGGACCGC. The remainder of the rabbit PXR 5'- and 3'-coding sequence was obtained by nested PCR using oli-

noroligonucleotides within the 861-bp internal rabbit PXR se-

quence and oligonucleotides within 5gt11, which flank the EcoRI cloning site. The oligonucleotides used for the nesting were: Nest 1: 5' CTAGAGCTCATCCGTTCCTGC and 5' AGGTGTTGACGGCTGAGGAAG; Nest 2: 5' AGCAGCACACCTGGAGCATG and 5' CATCTAGGCCGTCCTCATCAG. After obtaining the full-length rabbit PXR sequence by this method, the entire coding region was amplified and cloned again from rabbit liver cDNA to confirm the original sequence. The prim-

ers used in this step contained flanking EcoRI restriction sites for subcloning the PCR product into the mammalian expres-

vector pSG5 as well as a consensus Kozak sequence

modifications as previously described (39). In most cases,

Hepatocytes were isolated from human liver tissue obtained as surgical biopsy samples or from rejected donor livers by

were dispersed from the digested liver in DMEM containing

Hepatocytes were isolated from normal rabbit tissues using Trizol

isolated using Trizol reagent (Life Technologies, Inc.). Rabbit

RNA was isolated from normal rabbit tissues using Trizol reagent. Total RNA from each sample (10 µg) was resolved on a 2.2 m formaldehyde denaturing gel. The gels were stai-


Expression of Recombinant Human PXR LBD

The LBD of human PXR (amino acids 130–434) was expressed as an amino-terminal polyhistidine-tagged fusion protein. The PXR LBD was subcloned into the pRSETa bacterial expression vector (Invitrogen, San Diego, CA). Sequencing encoding a polyhistidine tag derived from an N-terminal PCR primer (MKGGHHHHHHHG) was fused in frame to the PXR LBD. To enhance its solubility, the PXR LBD was coexpressed with an 88-amino acid fragment of SRC-1 (42, 43). The human SRC-1 expression construct was created by insertion of a fragment of SRC-1 encoding amino acids 623–710 (43) flanked by Adel-BamHI restriction sites into the pACYC184 vector (New England Biolabs, Inc., Beverly, MA) containing the T7 promoter region of the pRSETa vector (Invitrogen). PCR primers were designed to amplify an 88-amino acid region of the human SRC-1 gene (p160), which encodes two of the LXLL motifs (motifs 1 and 2) along with an N-terminal tag (MKK). The resulting SRC-1/pACYC184 plasmid was cotransformed with PXRLBD/pRSETa into the BL21(DE3) E. coli strain. One-liter shake flask liquid cultures containing standard Luria-Bertani (LB) broth with 0.05 mg/ml ampicillin and 0.05 mg/ml chloramphenicol were inoculated and grown at 22°C for 24 h. The cells were induced with 0.05 mM isopropyl β-d-thiogalactopyranoside for 4–6 h at 22°C after which the cells were harvested by centrifugation (20 min, 3,500 × g, 4°C). The cell pellet was resuspended in 250 ml buffer A (50 mM Tris-Cl pH 8.0, 250 mM NaCl, 50 mM imidazole, pH 7.5). Cells were sonicated for 3–5 min on ice and the cell debris was removed by centrifugation (45 min, 20,000 × g, 4°C). The cleared supernatant was filtered through a 0.45 μm cutoff filter and loaded on to a 50 ml Ni2+ -charged ProBond Chelation resin (Invitrogen). After washing to baseline with buffer A, the column was washed with buffer A containing 125 mM imidazole, pH 7.5. The PXRLBD/SRC-1 complex was eluted from the column by increasing the concentration of imidazole to 500 mM (buffer A with 300 mM imidazole, pH 7.5). Column fractions were pooled and concentrated using Centri-prep 30K (Amicon, Beverly, MA) units. The protein was subjected to size exclusion using a column (26 mm × 40 cm) packed with Sepharose S-75 resin (Amersham Pharmacia Biotech, Piscataway, NJ) preequilibrated with 20 mM Tris-Cl, pH 8.0, 0.05 mg/ml ampicillin and 0.05 mg/ml chloramphenicol and added to the wells in 1 μl aliquots. $[^3]H$SR12813 was added to a final concentration of 10 nM. The plates were shaken momentarily to ensure complete mixing. After a 2-h incubation at room temperature, the plates were counted on a Packard TopCount, which was programmed to compensate for color quenching.

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