Simultaneous Substitution of Phenylalanine-305 and Aspartate-318 of Rat Pregnan X Receptor with the Corresponding Human Residues Abolishes the Ability to Transactivate the CYP3A23 Promoter

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ABSTRACT

The pregnane X receptor (PXR) is a key regulator on the expression of genes involved in the elimination of chemicals. As one of the most divergent members in the nuclear receptor family, PXR is activated in a highly species-dependent manner by certain chemicals. Pregnenolone 16α-carbonitrile (PCN), a glucocorticoid antagonist, efficaciously activates rodent but not human PXR. This study was undertaken to investigate the structural basis for PCN-mediated activation of rat PXR. A series of rat-human chimeric PXRs were prepared to gradually replace the ligand-binding domain of human PXR with the corresponding rat sequence at an increasing length of 20 residues. Cotransfection experiments established that region 306–326 acted as a transitional conjunction from none to full PCN responsive status. Site-directed mutagenesis study identified two residues (Phe-305 and Asp-318) that were critical in supporting PCN-mediated activation, and simultaneous substitution of both residues abolished the ability of rat PXR to transactivate the CYP3A23 promoter. In addition, substitutions on Phe-305, Asp-318, or both markedly reduced the basal transcriptional activity, and the reduction occurred with the CYP3A4 but not CYP3A23 promoter. Further study with CYP3A4 and CYP3A23 hybrid reporters demonstrated that the region harboring the distal PXR element in the CYP3A4 promoter mediated the repressive activity. PXR has been shown to interact with corepressors in the absence of ligand. The decreased responsiveness toward PCN and reduced basal transcriptional activity suggest that Phe-305 and Asp-318 are involved in both ligand-binding and corepressor interactions.

Metabolism and transport are considered the primary defense systems against chemicals from both foreign and endogenous sources (Brinkmann and Eichelbaum, 2001; Shou et al., 2001; Venkatakrishnan et al., 2001; Skatrud, 2002). Metabolism involves chemical modifications and usually leads to increased polarity of lipophilic compounds, thus favoring excretion into urine and feces. Active transport such as efflux moves compounds against chemical and concentration gradients, thus reducing intracellular concentrations or absorption of chemicals. Therapeutic agents, so-called intended chemicals, are also eliminated through transport and metabolism, and the elimination rate is directly related to their therapeutic effectiveness (Krishna and Mayer, 2000; Billard, 2003; Jansen et al., 2003). In addition, transport and metabolism are the most common sources for drug-drug interactions when two or more drugs are administered simultaneously. There are three major types of interactions based on the nature of interactions. Drugs competitively inhibit each other when their elimination is mediated by the same transporter/metabolizing enzyme (e.g., omeprazole versus diazepam) (Jones et al., 2004). Noncompetitive inhibition may occur with a drug that inactivates an enzyme, which is responsible for the metabolism of a coadministered drug (e.g., macrolide antibiotics versus terfenadine) (Yap and Camm, 2002). The third type of drug-drug interaction results from altered expression of an enzyme/transporter, leading to altered pharmacokinetics for all drugs that are eliminated by this enzyme/transporter (Hollenberg, 2002; Rushmore and Kong, 2002; Niemi et al., 2003).

Expression of drug-metabolizing enzymes/transporters is largely regulated at the transcriptional level (Blumberg et al., 1998; Kliwer et al., 1998; Goodwin et al., 2002; Kast et al., 2002; Rushmore and Kong, 2002). The pregnane X receptor (PXR), also called the steroid and xenobiotic receptor, has been recognized as a key regulator that mediates the induc-

ABBREVIATIONS: PXR, pregnane X receptor; PCN, pregnenolone 16α-carbonitrile; rPXR, rat pregnane X receptor; DDE, 1,1-dichlorodiphenyl-dichloroethylene; DR3, direct repeat spaced by three nucleotides; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide.
tion of many chemical elimination genes such as CYP3A4 and multidrug-resistant-1. PXR structurally belongs to a family of the nuclear receptors and regulates the transcription of target genes in a ligand-dependent manner (Goodwin et al., 2002). Unlike many other nuclear receptors, PXR has a large ligand-binding pocket that is spherical in shape, extremely hydrophobic, and expandable (Watkins et al., 2001, 2003a,b). Such structural features allow PXR to interact with a wide range of structurally dissimilar chemicals, including drugs (e.g., paclitaxel), herbal supplements (e.g., hyperforin), pesticides (e.g., trans-nonachlor), and endogenous compounds (e.g., bile acids) (Kliwer and Willson, 2002). PXR-null mice exhibit defects in the induction of drug-metabolizing enzymes and transporters in response to prototypical PXR activators, and these mice are hypersensitive to chemicals (e.g., zoxazolamine) that are substrates for the induced enzymes (e.g., CYP3A) (Xie et al., 2000, 2001; Staudsonger et al., 2001). Chemicals that exhibit species-dependent induction also show species-specific activation of PXR (e.g., rodent and human), and the species-specific induction is not observed in the mice that harbor a human PXR transgene (so-called humanized mice) (Xie et al., 2000).

Rifampicin and pregablenone 16α-carbonitrile (PCN) are the most studied inducers in terms of species-dependent activity (Schutz et al., 1984; Strolin Benedetti and Dostert, 1994; Moore et al., 2002; Niemi et al., 2003). Rifampicin is a potent inducer in humans and an efficacious activator of human PXR, and the same is true with PCN in rodents (Kliwer et al., 1998; Lehmann et al., 1998). Rifampicin differs markedly from PCN in terms of general chemical properties. Rifampicin is less hydrophobic and has a markedly larger molecular weight than PCN (mol. wt., 823 versus 342). Therefore, it is expected that rifampicin and PCN are not mutually exclusive in terms of PXR activation. In support of this notion, a human PXR mutant containing a substitution of the corresponding rat residue (L509F) shows decreased activity toward rifampicin but no changes toward PCN (Tirona et al., 2004). A human PXR mutant (Q285I) increases the ability to respond to PCN accompanied by decreased responsiveness to rifampicin; however, the corresponding rat PXR (rPXR) mutant with a reversed substitution (L502Q), although less responsive to PCN, shows no increases in responding to rifampicin (Östberg et al., 2002). Finally, rabbit PXR is markedly activated by both PCN and rifampicin (Moore et al., 2002).

The present study was undertaken to investigate the structural basis for PCN-mediated activation of rat PXR. Experiments with chimeric PXRs and site-directed mutagenesis identified two residues (Phe-305 and Asp-318) that were critical in supporting PCN-mediated activation, and simultaneous substitution of both residues completely abolished the ability to transactivate the CYP3A23 promoter. In addition, substitutions of Phe-305, Asp-318, or both markedly reduced the basal transcriptional activity, and the reduction occurred in the CYP3A4 but not the CYP3A23 promoter. PXR has been shown to interact with corepressors in the absence of ligand. The decreased responsiveness toward PCN and reduced basal transcriptional activity suggest that Phe-305 and Asp-318 are involved in both ligand-binding and corepressor interactions.

Materials and Methods

Chemicals and Supplies. DDE, dexamethasone, Hanks’ balanced salt solution, hyperforin, nifedipine, and PCN were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco’s modified Eagle’s medium, LipofectAMINE, and Plus reagent were purchased from Invitrogen (Carlsbad, CA). Kits for luciferase detection and the null Renilla luciferase plasmid were obtained from Promega (Madison, WI). Delipidated and normal fetal bovine sera were obtained from Hyclone Laboratories (Logan, UT), and the goat anti-rabbit IgG conjugated with alkaline phosphatase was obtained from Pierce (Rockford, IL). Nitrocellulose membranes were obtained from Bio-Rad (Hercules, CA). Unless otherwise specified, all other reagents were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Plasmid Constructs. The CYP3A23-DR3 reporter was constructed by inserting four copies of the CYP3A23 response element (5’-GATCATAGCTTATGAAGTCTATAGATC-3’) into the pGL3 promoter luciferase vector. The CYP3A23 promoter reporter (CYP3A23-Luc) was prepared by inserting the PCR-amplified genomic fragment (−1445 to +74) into the pGL3 promoterless luciferase vector (Huss and Kasper, 2000). The CYP3A4 promoter reporter (CYP3A4-DP-luc) containing fused fragments (−362 to +53 and −7836 to −6093) was described elsewhere (Song et al., 2004). The reporters are diagrammatically presented in Fig. 1A. The expression constructs encoding rat or human PXR were described previously (Zhang et al., 1998; Song et al., 2004). The chimeric PXR constructs were prepared by PCR-mediated site-directed mutagenesis as described in Fig. 1B. To facilitate the preparation of the chimera constructs, the rat PXR plasmid was subjected to site-directed mutagenesis to introduce two restriction endonuclease sites (BamHI and EcoRV). The BamHI site encodes residues 147 and 148, whereas the EcoRV site encodes residues 413 and 414. The resultant construct, designated rPXR<sub>147R/B414I</sub>, contained two amino acid substitutions (residues 147 and 414). The same endonuclease sites were introduced into the chimeric fragments through the S1 and A2 primers during PCR amplification (Fig. 1B). The chimeric fragments were digested with BamHI/EcoRV and ligated to the rPXR<sub>147R/B414I</sub> plasmid pretreated with the same enzymes. The rat-human chimeric PXRs were prepared to gradually replace the ligand-binding domain of human PXR with the corresponding rat sequence at an increasing length of 20 residues (Fig. 2A).

The fusion PCR method was also used to prepare constructs that contained multiple substitutions (e.g., rPXR<sub>147R/B414I/L305F/D318E</sub>); however, the rat PXR plasmid was used as the template for the initial amplification of both PCR fragments. All constructs were subjected to sequencing analyses.

Site-Directed Mutagenesis. The substitution mutants were prepared with a QuickChange site-directed mutagenesis kit as described previously (Li et al., 2003). Complementary oligonucleotides were synthesized to introduce a substitution. The primers were annealed to the rat PXR construct and subjected to a thermocycler for 15 cycles. The resultant PCR-amplified constructs were then digested with DpnI to remove the nonmutated parent construct. The mutated PCR-amplified constructs were used to transform XL1-Blue bacteria. Two rounds of site-directed mutagenesis were performed to generate double substitutions: rPXR<sub>147R/L414I</sub> and F<sub>305L/D318E</sub>. All mutated constructs were subjected to sequencing analysis to confirm the desired mutation being made without secondary mutations.

Transient Cotransfection Experiment. Cells (CV-1 and HepG2) were plated in 24-well plates in Dulbecco’s modified Eagle’s medium supplemented with 10% delipidated fetal bovine serum at a density of 8 × 10<sup>4</sup> cells per well. Transfection was conducted by lipofection with LipofectAMINE and Plus reagent as described previously (Song et al., 2004). Transfection mixtures contained 200 ng of a PXR plasmid, 100 ng of a reporter plasmid, and 10 ng of null Renilla luciferase plasmid. Cells were transfected for 4 h, and the medium was changed again with the same medium containing a chemical or solvent DMSO (final concentration, 0.1%). The transfected cells were
incubated for additional 24 h and washed once with phosphate-buffered saline and collected by scraping. The collected cells were subjected to two cycles of freeze/thaw. The reporter enzyme activities were assayed with a Dual-Luciferase Reporter Assay System as described by the manufacturer. This system contained two substrates that were used to determine the activity of two luciferases sequentially. The firefly luciferase activity, which represented the reporter activity, was initiated by mixing aliquot of lysates (10 μl) with Luciferase Assay Reagent II. Then the firefly luminescence was quenched, and the Renilla luminescence was simultaneously activated by adding Stop and Glo Reagent to the sample tube. The firefly luminescence signal was normalized based on the Renilla luminescence signal, and the ratio of normalized luciferase activity from chemical over DMSO treatment served as -fold induction.

Western Analysis. Cell lysates (8 μg) were resolved by 7.5% SDS-polyacrylamide gel electrophoresis in a minigel apparatus and transferred electrophoretically to nitrocellulose membranes. After nonspecific binding sites were blocked with 5% nonfat milk, the blots were incubated with an antibody against rat or human PXR. The antibodies were raised against a peptide derived from respective PXR. The sequence of the peptide from rat PXR was N-CEEAD-SALEEPINVEE-C, whereas the sequence of the peptide from human PXR was N-CELPEVLQAPS-C. The production and purification of antibodies were described previously (Sachdeva et al., 2003). The primary antibodies were subsequently localized with the goat anti-rabbit IgG conjugated with horseradish peroxidase (Ma et al., 2004). Horseradish peroxidase activity was detected with a chemiluminescent kit (SuperSignal West Pico; Pierce). The chemiluminescent signal was captured by KODAK Image Station 2000, and the relative intensities were quantified by KODAK 1D Image Analysis Software.

Other Analyses. Protein concentrations were determined with a bicinchoninic acid assay (Pierce), with albumin as standard. Data are presented as mean ± S.D. of at least three separate experiments, except where results of blots are shown in which case a representative experiment is depicted in the figures. Comparisons between two values were made with Student’s t test at p < 0.05.

Results

Region306–326 Functions as the Switching Conjunction for PCN Responsiveness. Antiglucocorticoid PCN, a prototypical inducer of CYP3A enzymes in rodents, efficaciously activates rat but not human PXR. The present study was undertaken to locate residues that support PCN-mediated activation. A series of rat-human chimeric PXRs were prepared to gradually replace the ligand-binding domain of human PXR with the corresponding rat sequence at an increasing length of 20 residues (Fig. 2A, left). The chimeric ligand-binding domains were generated with fusion PCR as described in Fig. 1B. The responsiveness of chimeric PXRs toward PCN was determined by cotransfection experiments.
with a reporter containing four copies of PXR DR3 response element (CYP3A23-DR3-Luc) (Fig. 1A).

The activation of chimeric PXRs by PCN is summarized in Fig. 2A. No evident activation was detected with human PXR or chimeric PXR rh147, which contained almost the entire ligand-binding domain of human PXR (Fig. 2A). In fact, all chimeric PXRs containing a replaced sequence up to residue 306 remained nonresponsive to PCN. In contrast, the replacement of an additional 20 residues (rh326) resulted in an abrupt increase in PCN responsiveness. Actually, a similar magnitude of the activation was detected with all chimeric PXRs that had a replaced sequence equal to or longer than that in the chimeric PXR rh326 (Fig. 2A). It should be emphasized that rPXRL147R/T414I, containing two amino acid substitutions, responded to PCN in much the same way as the wild-type PXR. In addition, some chimeric PXRs, notably rh326, rh346, and rh366, showed higher basal activity (DMSO) (Fig. 2A).

To determine whether chimeric PXR constructs produced similar levels of PXR protein, Western analysis was performed with an antibody against rat or human PXR. As shown in Fig. 2B, transfection of all PXR constructs resulted in a comparable level of expression. In addition to human PXR, three chimeric PXRs, including rh147, rh166, and rh186, were detected by the antibody against human PXR. The peptide used for the preparation of the anti-human PXR antibody was derived from residues 182 to 193 and thus overlapped with the chimeric rh186 by only four residues (Zhang et al., 1999). The detection of this chimeric PXR by this antibody suggests that these four residues are sufficient to form an immunogenic epitope (Fig. 2B).

Quadruple Mutants PXR A302S/F305L/P308T/N309A and PXR K314Q/D318E/L320M/M321L No Longer Respond to PCN.

The study with chimeric PXRs suggested that region 306–326 acts as a transitional conjunction from none to full PCN responsive status. To further support this conclusion, concentration-dependent activation and site-directed mutagenic studies were performed. For the concentration-dependent activation experiment, the wild-type and two chimeric PXRs were tested, including rh306 and rh326. As shown in Fig. 3A, both the wild-type and chimeric rh326 PXRs were activated in a concentration-dependent manner. The wild-type PXR was activated slightly higher than rh326 at lower concentrations (<10 μM), whereas the opposite was true when higher concentrations (≥10 μM).
concentrations were used (≥10 μM) (Fig. 3A). In contrast, no activation was detected with rh306, regardless of the concentrations. These results further support the conclusion that region306–326 plays a transitional role in PCN response.

Site-directed mutagenesis was performed to determine whether the transitional role occurs with the wild-type PXR. Based on sequence alignment analysis, there are seven residues that differ between rat and human PXR (Fig. 3B, bold). Two quadruple substitution mutants, PXRA302S/F305L/P308T/N309A and PXRK314Q/D318E/L320M/M321L, were prepared to replace the nonconserved amino acids with corresponding human residues. Residues 302 and 305 were included in the mutagenic study because of their proximity to region306–326 and previously implicated role in PCN-mediated activation (Tirona et al., 2004). Residue 325 (cysteine), on the other hand, was excluded from the quadruple substitutions based on our initial observation that this residue played no role (described below). Similarly, the transactivation activity of the quadruple mutants was determined by co-transfection experiments. As expected, increasing concentrations of PCN caused proportional increases in the activation of rat PXR (Fig. 3C). In contrast, none of the quadruple mutants showed evident activation, even with the highest concentration of PCN (50 μM) (Fig. 3B). Western analysis detected comparable expression of the quadruple mutants as the wild-type PXR (data not shown).

Simultaneous Substitution of Phenylalanine-305 and Aspartate-318 Abolishes the Responsiveness to PCN. The study with the quadruple mutants suggested that one or more residues in each mutant are critical in supporting PCN-mediated activation. To directly test this possibility, a single residue substitution was made from rat to human. Among nine mutants, only D318E and F305L exhibited a marked reduction on the PCN-mediated activation (Fig. 4A). The reduced activation with F305L was more profound than that with D318E (85 versus 60%). The third highest reduction

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**Fig. 3.** Concentration-activation curves of rPXR, rh306, rh326, PXRA302S/F305L/P308T/N309A, and PXRK314Q/D318E/L320M/M321L. A, concentration-activation experiment with rPXR, rh306, and rh326. CV-1 cells were transiently transfected with construct mixtures containing 200 ng of rPXR or a chimeric PXR (rh306 and rh326), 100 ng of CYP3A23-DR3-Luc, and 10 ng of null-luciferase plasmid. The transfected cells were treated with PCN (0–50 μM) or the same volume of DMSO for 24 h. Luciferase activities of the reporter were determined and normalized based on the Renilla luminescence signal. Data represent the mean of assays in triplicate ± S.D. B, alignment analysis of rat and human PXRs from residues 301 to 326. C, activation of quadruple mutants PXRA302S/F305L/P308T/N309A and PXRK314Q/D318E/L320M/M321L. CV-1 cells were transiently transfected with construct mixtures containing 200 ng of rPXR or a quadruple mutant, 100 ng of CYP3A23-DR3-Luc, and 10 ng of null-luciferase plasmid. The transfected cells were then treated with PCN (0–50 μM) or the same volume of DMSO for 24 h. Luciferase activities of the reporter were determined and normalized based on the Renilla luminescence signal. Data represent the mean of assays in triplicate ± S.D.
was detected with mutant K314Q (~30%). The rest of the mutants showed some reduction (10–20%) except A302S, which was activated comparably as the wild-type PXR (Fig. 4A). Apparently, substitutions on these residues caused no changes on the expression (Fig. 4A).

The profound reduction with F305L and D318E underscored the importance of Phe-305 and Asp-318 in supporting PCN-mediated activation. We next tested whether these two residues worked additively on responding to PCN. A mutant (F305L/D318E) was prepared to simultaneously substitute both residues. As shown in Fig. 4B, the double mutant F305L/D318E no longer responded to PCN, regardless of the concentrations of PCN (0–50 μM). In contrast, F305L and D318E were activated in a concentration-dependent manner, although the overall magnitude was markedly lower than that with the wild-type PXR (Fig. 4B). Even between F305L and D318E, marked differences were detected, with F305L being activated to a much lesser extent (Fig. 4B). Interestingly, both F305L and D318E exhibited a relatively lesser reduction with increasing PCN concentrations. For example, at 50 μM, D318E showed a reduction of ~30%, which contrasted to a 60% reduction at 10 μM (Fig. 4B). It should be emphasized that additional mutants were prepared to contain double or triple substitutions as specified in either PXR_A302S/F305L/D318E or PXR_K314Q/D318E/L320M/M321L. However, no mutants showed a significant reduction unless one of the residues was Phe-305 and Asp-318, and mutants containing triple substitutions such as PXR_D318E/L320M/M321L no longer responded to PCN, as seen with F305L/D318E (data not shown), suggesting that other residues work cooperatively with Phe-305 and Asp-318 and contribute to the overall magnitude of the activation.

**Chemical-Dependent Alteration among Mutants**

F305L, D318E, and F305L/D318E. The inability of F305L/D318E to elicit any transactivation suggested that both residues are critical in supporting PCN-mediated activation. Next we tested whether single or double substitutions altered sensitivity toward other chemicals such as nifedipine, DDE, and hyperforin. The selection of the chemicals was largely based on their differences on size and lipophilicity. Based on the activation of the wild-type PXR, other chemicals tested were less potent than PCN (Fig. 5). For example, when assayed at 50 μM, DDE and nifedipine activated the wild-type PXR by only ~10% and 35% of that elicited by PCN, respectively (Fig. 5, A–C). The mutants, on the other hand, exhibited differential changes depending on a chemical. PCN
and nifedipine caused markedly less activation of all mutants, and the reduced activation was more profound with nifedipine. For example, $D_{318E}$ showed a 35% reduction with PCN but 70% with nifedipine when they were assayed at 50 μM (Fig. 5, A and B). In contrast to the reduced activation by PCN and nifedipine, DDE slightly increased the activation of both $F_{305L}$ and $D_{318E}$ (Fig. 5C). With hyperforin, $F_{305L}$ was activated slightly higher than the wild-type PXR (e.g., 4-fold versus 3-fold at 5 μM), whereas neither $D_{318E}$ nor $F_{305L}/D_{318E}$ showed any evident activation by this natural compound (Fig. 5D).

Promoter-Dependent Alteration among $F_{305L}$, $D_{318E}$, and $F_{305L}/D_{318E}$. We next examined the transactivation activity of the mutants toward native promoters regulated by PXR. Two promoter reporters were tested, including CYP3A23-Luc and CYP3A4-DP-Luc. The CYP3A23-Luc reporter contained a 1519-base pair genomic fragment from the proximal promoter region, where a DR3 element is present (Fig. 1A). The CYP3A4-DP-Luc reporter contained a DR3 element in the distal region (−7836 to −6093) and an everted repeat spaced by six nucleotides element in the proximal promoter (−362 to +53) (Fig. 1A). Both elements are required for the maximum activation in response to a PXR ligand (Goodwin et al., 1999; Song et al., 2004). In addition to CV-1, hepatoma-derived cell line HepG2 was used for the cotransfection experiment. It should be emphasized that the element reporter (CYP3A23-DR3-Luc) provided a simplified model and indicated a direct functional outcome as a result of the interactions between PXR and its element, whereas the promoter reporters represented a more physiologically related scenario and better indicated the responsiveness of the native genes (CYP3A23 and CYP3A4), particularly when the hepatic line HepG2 was used.

The results from the transactivation of various reporters are summarized in Fig. 6. Between two cell lines, HepG2 cells generally supported higher activation than CV-1 cells. Among three reporters, the highest transactivation was detected with CYP3A23-DR3-Luc, and the lowest was detected with CYP3A23-Luc. CYP3A23-DR3-Luc and CYP3A23-Luc showed similar transactivation patterns by four PXRs in both CV-1 and HepG2 cells (Fig. 6, A and B). In contrast, the transactivation of the CYP3A4-DP-Luc reporter exhibited profound differences depending on the type of cells. In CV-1 cells, the wild-type, $F_{305L}$, and $D_{318E}$ PXRs were activated to the same extent (Fig. 6C). In contrast, in HepG2 cells, a profound differential activation was detected. The wild-type PXR and $D_{318E}$ mutant were activated to a comparable extent, whereas a 40% reduction was detected with $F_{305L}$. The differences on the transactivation of the CYP3A4-DP-Luc reporter suggest that HepG2 and CV-1 cells support differential expression of genes that participate in the regulation of CYP3A4 expression (in addition to PXR).

Interestingly, in both CV-1 and HepG2 cells, all PXR mutants exhibited markedly lower basal activity (DMSO-treated cells) than the wild-type PXR toward the CYP3A4-DP-Luc reporter (Fig. 6C). As a result, the overall activation of mutants $F_{305L}$ and $D_{318E}$ was actually higher than that with the wild-type PXR when the data were expressed as fold activation (Fig. 6D). For example, $F_{305L}$ was activated lower than the wild-type PXR by 35% based on the normal-
ized luciferase activity (Fig. 6C), whereas it was actually activated markedly higher based on the -fold induction (40-versus 19-fold) (Fig. 6D). Tirona et al. (2004) recently reported that mutant F305L was activated higher than the wild-type PXR by PCN. They used a CYP3A4 reporter, and the data were expressed as -fold activation. Therefore, it is likely that the reported higher -fold activation was actually due to decreased basal activity (Tirona et al., 2004).

The Distal Region (–7836 to –6093) in the CYP3A4 Promoter Mediates Transcriptional Repression. The CYP3A4-DP-Luc was prepared to contain the proximal promoter and a distal region (Fig. 1A). Goodwin et al. (1999) reported that the proximal region and part of the distal region are required for maximum activation; however, the lack of the distal region no longer responds to PXR-mediated transactivation. We next examined whether the distal region is responsible for the decreased basal activity. The distal region was released from the CYP3A4-DP-Luc reporter and inserted into the CYP3A23-Luc reporter (the upstream of the CYP3A23 proximal promoter). The parent reporter (CYP3A23-Luc) and the reporter containing the distal region (CYP3A23 + 3A4-D-Luc) were tested for the differential ability to respond to PXR-mediated transactivation. Mutant F305L was also included in this study to determine whether this mutant differs from the wild-type PXR in modulating the basal activity of these reporters. Similarly, the data were expressed as normalized luciferase activities and -fold activation (Fig. 7).

Based on the normalized luciferase activities, the CYP3A23 + 3A4-D-Luc reporter exhibited markedly lower activation than the CYP3A23-Luc reporter in response to either wild-type PXR or mutant F305L (Fig. 7, A and C). As expected, the basal activity of the CYP3A23 + 3A4-D-Luc reporter was much lower than that of the CYP3A23-Luc reporter (DMSO lanes, labeled as no PCN). Interestingly, the decreased basal activity was observed with both wild-type PXR and mutant F305L, with the wild-type PXR showing a higher reduction (Fig. 7, A and C). Based on the -fold activation, both reporters were transactivated by PXR and F305L, although the CYP3A23 + 3A4-D-Luc reporter consistently exhibited a slightly or significantly decreased -fold activation depending on the concentrations of PCN (Fig. 7, B and D). In addition, the concentration-dependent activation curve by the wild-type PXR differed from that by F305L. At lower concentrations (<10 μM), the wild-type PXR caused higher transactivation than mutant F305L on both reporters, whereas the mutant F305L caused higher transactivation at Fig. 6. Transactivation of CYP3A23-DR3-Luc, CYP3A23-Luc, and CYP3A4-DP-Luc in CV-1 and HepG2 cells. A, transactivation of CYP3A23-DR3-Luc. CV-1 or HepG2 cells were transfected with construct mixtures containing 200 ng of rPXR, F305L, D318E, or F305L/D318E; 100 ng of CYP3A23-DR3-Luc; and 10 ng of null-luciferase plasmid. The transfected cells were treated with 10 μM PCN or the same volume of DMSO for 24 h. Luciferase activities of the reporter were determined and normalized based on the Renilla luminescence signal. Data represent the mean of assays in triplicate ± S.D. The same experiments were performed with CYP3A23-Luc (B) or CYP3A4-DP-Luc (C and D). The data with CYP3A4-DP-Luc were expressed as normalized luciferase activities (C) or -fold activation (D).
higher concentrations, notably at 50 μM (e.g., 8- versus 14-fold for CYP3A23-Luc) (Fig. 7, B and D).

Discussion

PXR is recognized as a key transcription regulator on the expression of genes involved in chemical elimination (Goodwin et al., 2002). Like many other nuclear receptors, PXR contains a DNA-binding domain and a ligand-binding domain. Based on the nucleotide substitution rate in the ligand-binding domain, PXR represents one of the most divergent members in the nuclear receptor family. As a result, certain chemicals activate PXR in a highly species-dependent manner (Goodwin et al., 2002). Antiglucocorticoid PCN, a prototypical inducer of CYP3A enzymes in rodents, efficaciously activates rat but not human PXR. In this study, we report that Phe-305 and Asp-318 play critical roles in supporting PCN-mediated activation, and simultaneous substitution of both amino acids with their human counterparts abolishes the ability to transactivate the CYP3A23 promoter. In addition, experiments with a series of rat-human chimeric PXRs have established that region306–326 functions as a transitional conjunction switching from none to full responsive status.

The transitional role of region306–326 in supporting PCN-mediated activation suggests that the N-terminal 188 residues of the ligand-binding domain (a total of 293 amino acids) determine the specificity toward PCN and its activation magnitude. In this study, the chimeric PXR rh326, containing approximately two thirds of the rat ligand-binding domain (N terminus), produces a similar concentration-dependent activation curve as rat PXR (Figs. 2A and 3A). Interestingly, the importance of this region in ligand specificity seems to be a general phenomenon among all PXRs. Rifampicin, for example, is an efficacious activator of human but not rat PXR. A rat-human chimeric PXR containing the N-terminal 184 residues of the rat ligand-binding domain remains nonresponsive to rifampicin as rat PXR (Tirona et al., 2004). Conversely, a human-rat PXR with a switched order of the chimeric fragments is markedly activated by this antibiotic. In contrast to a single transitional region toward PCN (Fig. 2A), multiple transitional regions are located with rifampicin, and they are all present in the N-terminal two thirds of the ligand-binding domain (Tirona et al., 2004).

Region306–326 has several structural features that likely support the transitional role. Based on X-ray crystallographic studies on human PXR, part of this region forms a flexible loop linking the C terminus of β4 to the N terminus of α7 (Watkins et al., 2001). The β4, along with other four-stranded β sheets, forms a wall that constitutes one side of the binding cavity. In particular, the flexible loop is involved in the formation of the entrance of the cavity and likely facilitates the expansion of the binding pocket (Watkins et
al., 2001). Apparently, the flexibility of this loop is critical for PXRs to accommodate ligands with various sizes, such as rifampicin (mol. wt., 823) and PCN (mol. wt., 342). Another important feature of this loop is the ability to adopt ligand-induced conformation (Watkins et al., 2003b). In the hyperforin-PXR structure, part of the loop (residues 314 to 318, based on rat PXR) adopts a helical conformation, whereas such an adoption does not occur in the PXR/SR12813 complex (Watkins et al., 2001, 2003b). The chemical-dependent adoption on the conformation provides another structural basis for the broad ligand specificity associated with PXR.

The flexibility of this region and the expandability of the entire cavity are likely restricted by interactions with other proteins. PXR dimerizes with RXRs, and only the heterodimers bind to the PXR element (Kliewer et al., 1998). In addition, PXR has been shown to interact with corepressors and coactivators, and such interactions depend on the presence of a ligand (Gonzalez and Carlberg, 2002; Li et al., 2002). Ligand binding triggers dissociation from a corepressor and initiates association with a coactivator. X-ray crystallographic analysis reveals several structural differences on PXR when cocrystallized with SR12813 and a coactivator peptide (Watkins et al., 2003a). In the presence of the peptide, PXR seems to form homodimers instead of monomers. Interactions with this peptide also affect the binding mode of SR12813. In the PXR/SR12813 peptide complex, SR12813 lies in the cavity in a single rather than three different orientations observed in the absence of the peptide (Watkins et al., 2001, 2003a). Apparently, PXR and its directly associated proteins represent only part of the transcriptional regulatory complex present in a promoter. It is conceivable that other proteins in a promoter likely affect ligand binding and ultimately the transactivation activity of PXR.

The residues in transitional region306–326 likely make direct contact with a ligand, which in turn alters the sensitivity of PXR. Based on X-ray crystallographic structures, hyperforin and SR12813 make direct contacts with residues 320 and 321, respectively (Watkins et al., 2001, 2003b). Interestingly, substitution of either residue causes only a moderate reduction (~20%) on the sensitivity toward PCN (Fig. 4A). In contrast, substitution of residues 305 or 318 (F305L and D318E) reduces the transactivation activity by 85 and 60%, respectively (Fig. 4A). X-ray crystallographic study reveals that glutamate-318 is involved in the formation of a salt bridge with Arg-410 (Watkins et al., 2001). This residue is conserved in rat PXR and is expected to form the same salt bridge with aspartate-318 (Zhang et al., 1999). However, an Arg-Asp salt bridge likely brings closer the structures where each residue resides (the flexible loop and α10). Given the fact that Glu-318 is located at the entrance of the binding cavity, it is conceivable that the substitution of aspartate with glutamate likely enlarges the entrance and increases the sensitivity toward a larger ligand. However, contrary to this prediction, D318E is activated much lower than the wild-type PXR by hyperforin, which is significantly larger than PCN (Figs. 5D and 8).

The size of a ligand, in reference to the size of the entrance, plays only a contributing role to the overall magnitude of

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**Fig. 8.** Chemical structure of PCN, nifedipine, DDE, and hyperforin.
activation. Phe-305 (based on rat PXR) is one of the 28 residues that line the inner surface of the binding cavity (Watkins et al., 2001). Substitution of this aromatic residue with a leucine likely decreases the hindrance caused by the bulky aromatic ring; however, F305L exhibits reduced activation toward PCN with only a slight increase toward DDE and hyperforin (Fig. 5). It is likely that a relatively bulky residue at the entrance of the cavity imposes certain restrictions and thus properly orient the entry of a ligand, particularly with small chemicals. Another possibility is that a residue (e.g., Leu-308 in human PXR) in region 306–326 works in concert with other residues to achieve the overall biological activity. In support of the notion, we have demonstrated that quadruple mutants PXR_A302S/F305L/P308T/N309A and PXR_K314Q/D318E/L320M/M321L show no activation activity toward PCN, although individual substitution of these residues cause only slight or moderate reduction, with the exception of Phe-305 and Asp-318 (Figs. 3C and 4A). It should be emphasized that the substitution of these residues in rat PXR with human counterparts generally results in reduced activation (Fig. 4A), suggesting that these amino acids indeed contribute to the insensitivity of human PXR toward PCN.

It is interesting to notice that the distal region of the CYP3A4 promoter mediates repressive activity on the basal transcription. In this study, we have demonstrated that the fusion of this region to the CYP3A23-Luc reporter results in marked decreases on the basal transactivation (Fig. 7, A and C). The precise mechanism on the decreased basal activity remains to be determined. Based on the fact that PXR binds to corepressors in the absence of a ligand (Gonzalez and Carlb erg, 2002), it is likely that the distal region provides a better platform for PXR and corepressor interactions. Alternatively, the distal region serves a binding site for a corepressor, and the binding of this repressor and the corepressing function are modulated by PXR-DNA interactions. In addition, the proximal promoter seems to modulate the interactions in the distal region, which depend on the type of PXR. For example, the wild-type PXR is more effective than mutant F305L in reducing the basal activity of the CYP3A23+3A4-d-Luc reporter (Fig. 7, A and C), whereas the opposite is true with the CYP3A4-DP-Luc reporter (Fig. 6D).

The decreased basal activity likely complicates the interpretation of the role of a residue in basal and ligand-regulated transcription. Reporter activities are usually expressed as -fold activation or normalized activities. -Fold activation but not normalized activity takes into consideration both basal (DMSO-treated) and ligand-regulated activity (e.g., PCN). Interestingly, these two expression methods may in some cases lead to opposing conclusions, particularly when a residue is involved in both basal and ligand-regulated activities. For example, the wild-type PXR is more active than the mutant F305L in transactivating the CYP3A4-DP-Luc reporter based on the normalized luciferase activity (Fig. 6C), whereas the opposite is true based on the -fold induction (40 versus 19-fold) (Fig. 6D). As for the transactivation of CYP3A23-Luc and CYP3A23+3A4-d-Luc reporters, the wild-type PXR is more active than mutant F305L at low PCN concentrations (e.g., 5 and 10 μM), whereas the opposite is true at high concentrations (e.g., 50 μM) (Fig. 7, B and D). These findings suggest that high concentrations of PCN overcome the repression on the basal transcription mediated by this mutant, and Phe-305 is involved in both ligand and corepressor interactions.

References


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