

Regulation and Binding of Pregnane X Receptor by Nuclear Receptor Corepressor Silencing Mediator of Retinoid and Thyroid Hormone Receptors (SMRT)

David R. Johnson, Chia-Wei Li, Lih-Yow Chen,¹ Jagadish C. Ghosh, and J. Don Chen

Department of Pharmacology, University of Medicine & Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, New Jersey

Received March 30, 2005; accepted October 11, 2005

ABSTRACT

The pregnane X receptor (PXR) is an orphan nuclear receptor predominantly expressed in liver and intestine. PXR coordinates hepatic responses to prevent liver injury induced by environmental toxins. PXR activates cytochrome P450 3A4 gene expression upon binding to rifampicin (Rif) and clotrimazole (CTZ) by recruiting transcriptional coactivators. It remains unclear whether and how PXR regulates gene expression in the absence of ligand. In this study, we analyzed interactions between PXR and the silencing mediator of retinoid and thyroid hormone receptors (SMRT) and determined the role of SMRT in regulating PXR activity. We show that SMRT interacts with PXR in glutathione S-transferase pull-down, yeast two-hybrid, and mammalian two-hybrid assays. The interaction is mediated through the ligand-binding domain of PXR and the SMRTs'

nuclear receptor-interacting domain 2. The PXR-SMRT interaction is sensitive to species-specific ligands, and Rif causes an exchange of the corepressor SMRT with the p160 coactivator known as receptor-associated coactivator 3 (RAC3). Deletion of the PXR's activation function 2 helix enhances SMRT binding and abolishes ligand-dependent dissociation of SMRT. Coexpression of PXR with SMRT results in colocalization at discrete nuclear foci. Finally, transient transfection assays show that overexpression of SMRT inhibits PXR's transactivation of the *Cyp3A4* promoter, whereas silencing of SMRT enhances the reporter expression. Taken together, our results suggest that the corepressor SMRT may bind to and regulate the transcriptional activity of PXR.

CYP3A4 is a member of the cytochrome P450 superfamily of drug-metabolizing enzymes. CYP3A4 makes up a large portion of the total cytochrome P450 concentration in liver and intestine (Guengerich, 1999). CYP3A4 metabolizes a vast array of endogenous, therapeutic, and xenobiotic chemicals, resulting in detoxification of 50 to 60% of all prescribed therapeutic drugs. CYP3A4 protein activity can be significantly increased by treatments with a wide range of chemical inducers. Pregnane X receptor (PXR) or xenobiotic sensing

nuclear receptor (SXR; NR1I2) binds to the promoter of *Cyp3a4* gene and regulates its expression (Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998). PXR is a promiscuous nuclear receptor for xenobiotic chemicals and endogenous metabolites, and it coordinates hepatic responses with the constitutive androstane receptor (CAR, NR1I3) to prevent liver injury (Stedman et al., 2005). The human PXR (hPXR) is a 434-amino acid, 50-kDa protein primarily expressed in liver and intestine (Lehmann et al., 1998), the same organs that express CYP3A4. PXR heterodimerizes with the retinoid X receptor on several DNA elements, including direct repeats separated by three nucleotides, everted motifs separated by six nucleotides, and inverted motifs separated by six or eight nucleotides (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998;

J.D.C. is a Research Scholar of the Leukemia and Lymphoma Society. This work was supported by National Institutes of Health grant DK52542.

D.R.J. and C.-W.L. contributed equally to this work.

¹ Current affiliation: Baylor College of Medicine, Houston, TX.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.105.013375.

ABBREVIATIONS: PXR, pregnane X receptor; SXR, xenobiotic sensing nuclear receptor; CAR, constitutive androstane receptor; hPXR, human PXR; Rif, rifampicin; CTZ, clotrimazole; RU-486, mifepristone; mPXR, mouse pregnane X receptor; PCN, pregnenolone-16 α -carbonitrile; DEX, dexamethasone; SMRT, silencing mediator of retinoid and thyroid hormone receptors; ID1, nuclear receptor interacting domain 1; ID2, nuclear receptor interacting domain 2; AF1, activation function 1; AF2, activation function 2; aa, amino acid(s); GST, glutathione S-transferase; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; HSD, honestly significant difference; LSD, least significant difference; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; siRNA, small interfering RNA; hRAR, human retinoic acid receptor; LBD, ligand binding domain; DBD, DNA binding domain; shRNA, short hairpin RNA; LXR β , liver X receptor β ; Δ AF2, AF2 deletion; Δ AF1, AF1 deletion; PPAR, peroxisome proliferator-activated receptor; RAC3, receptor-associated coactivator 3.

Lehmann et al., 1998; Kast et al., 2002). Once activated, the PXR/retinoid X receptor heterodimer recruits nuclear receptor coactivators, such as the p160 proteins, to form a multiprotein complex that leads to the initiation of gene transcription (Kliewer et al., 1998; Lehmann et al., 1998).

PXR can be activated by a diverse group of chemical compounds. The xenobiotic activators include the antibiotic rifampicin (Rif) (Lehmann et al., 1998; Goodwin et al., 1999), the antimycotic drug clotrimazole (CTZ) (Bertilsson et al., 1998; Lehmann et al., 1998), the chemotherapeutic agent paclitaxel (Synold et al., 2001), the antigluco-corticoid RU-486 (Bertilsson et al., 1998), and environmental contaminants such as polychlorinated biphenols (Schuetz et al., 1998). Some PXR activators are dehydroepiandrosterone and metabolites (Ripp et al., 2002), the bile acid lithocholic acid (Staudinger et al., 2001), and the 5 β -pregnane-3,20-dione and other pregnane derivatives (Bertilsson et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998). Some of these chemicals produce a species-specific effect on CYP3A up-regulation via PXR activation. This is most apparent with Rif and CTZ, which activate hPXR but not mouse PXR (mPXR), whereas the pregnenolone-16 α -carbonitrile (PCN) and dexamethasone (DEX) activate mPXR but not hPXR (Lehmann et al., 1998; Jones et al., 2000; Xie et al., 2000a). Most of these chemical inducers have been well characterized as having up-regulated CYP3A4.

Whether PXR plays a role in regulating gene expression in the absence of ligand remains controversial. It was reported that the basal expression of the mouse *Cyp3a11* (homolog of the human *Cyp3a4*) was significantly increased in PXR knockout mice (Staudinger et al., 2001). However, such an increase was not observed in another knockout mouse line (Xie et al., 2000a). If PXR is capable of repressing gene expression in the absence of ligand, it is possible that transcriptional repression by PXR may be because of protein-protein interaction with nuclear receptor corepressors such as SMRT and/or N-CoR (Chen and Evans, 1995; Horlein et al., 1995; Park et al., 1999), which facilitate the formation of a nuclear protein complex that represses transcription by recruiting histone deacetylases (Ordentlich et al., 2001). To understand whether and how PXR regulates basal expression of *Cyp3a4*, we have determined the intrinsic transcriptional activity of PXR and examined its interaction with SMRT. SMRT is a 270-kDa nuclear protein that interacts with nuclear receptors by two receptor interacting domains, ID-1 and ID-2, which contain extended L-XXX-L-XXX-I/L corepressor motifs (Hu and Lazar, 1999; Wu et al., 2001).

In this study, we show that PXR and SMRT form a tight complex in vitro and in yeast and cultured mammalian cells, and ligand binding causes dissociation of this complex. PXR selectively interacts with the SMRT ID2 motif, and the AF2 helix of PXR is involved in the mechanism of SMRT dissociation. We found that PXR colocalizes with SMRT at discrete nuclear foci. Overexpression of SMRT also repressed PXR activation on CYP3A4 promoter, and SMRT knockdown using siRNA up-regulated PXR transcriptional activity. These results suggest that the corepressor SMRT may bind to and regulate the transcriptional activity of PXR.

Materials and Methods

Chemicals and Reagents. Rif, CTZ, PCN, DEX, and *all-trans* retinoic acid were purchased from Sigma (St. Louis, MO). The anti-SMRT monoclonal antibody was a generous gift from GeneTex, Inc. (San Antonio, TX). The anti-HA polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals used were of the highest grade.

Plasmids Constructs. Full-length pSG5-PXR Δ ATG was a generous gift from Steve Kliewer (Southwestern Medical Center, Dallas, TX). PXR was subcloned into pGBT9, pCMX-HA, and pCMX-Gal4 plasmids by restriction enzyme digestion (New England Biolabs, Beverly, MA). The Δ AF2 mutant (aa 1–422), C-terminal fragment (C-terminal, aa 141–434), and N-terminal fragment (N-terminal, aa 1–204) of hPXR were generated by PCR and subcloned into various vectors. The human SMRT τ (Park et al., 1999) corresponds to the SMRT τ isoform (GenBank accession number AY965853). The cSMRT, ID1, ID2, cSMRT-mID1, cSMRT-mID2, and cSMRT-mID1/2 were as described previously (Ghosh et al., 2002). The 13-kb CYP3A4-luc reporter was a generous gift of Christopher Liddle (University of Sydney at Westmead Hospital, Westmead, Australia).

GST Pull-Down Assay. The GST-fusion proteins of cSMRT, ID1, ID2, mID1, mID2, and mID1/2 were as described previously (Ghosh et al., 2002). The various nuclear receptor proteins were synthesized in rabbit reticulocyte lysate and radiolabeled with [35 S]methionine using the TNT Quick-Coupled Transcription/Translation kit (Promega, Madison, WI). Glutathione Sepharose bead-bound GST fusion protein (10 μ l) was blocked with 10 mg/ml bovine serum albumin (BSA) at room temperature for 5 min before mixing with BSA-blocked 35 S-labeled receptor proteins. Proteins were mildly shaken at 4°C overnight in binding buffer (20 mM HEPES, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl $_2$, 0.05% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM methionine, and 1 μ g/ml BSA). The protein mixture was washed three times with fresh binding buffer, and the beads were collected by centrifugation at 3000 rpm for 5 min. After boiling for 10 min in SDS sample buffer, bound beads were subject to SDS-polyacrylamide gel electrophoresis. Gels were then stained with Coomassie blue, dried, and detected by autoradiography.

Yeast Two-Hybrid Assay. The yeast Y190 cells were cotransformed with indicated plasmids and 4 μ g of single-stranded salmon sperm DNA according to the manufacturer's protocol (BD Biosciences Clontech, Palo Alto, CA). The transformed yeast cells were spread on selection plates and incubated at 30°C for 2 days. Colonies were picked from each plate and grown in tryptophan and leucine-deficient media for 48 h at 30°C. Two aliquots (100 μ l each) from each sample were taken and added to fresh selection media. For each sample, one aliquot received ligands and the other aliquot received vehicle control (DMSO). Samples were then shaken in a 30°C incubator for an additional 48 h. Yeast cells were then harvested in Z-buffer containing 0.27% β -mercaptoethanol and analyzed for β -galactosidase activity by liquid assay using O-nitrophenyl- β -galactopyranoside as substrate as described previously (Chen et al., 1996).

Statistical analyses were conducted by one-way analysis of variance (ANOVA) followed by post hoc pair-wise comparisons of sample means via the conservative Tukey's honestly significant difference (HSD) or the liberal least significant difference (LSD) test. The ANOVA procedure was performed by the SAS statistical software (SAS Institute, Inc. Cary, NC) or online at <http://faculty.vassar.edu/lowry/VassarStats.html> for samples of fewer than five groups.

Immunofluorescence Colocalization Analysis. COS-7 cells were seeded on coverslips in 12-well plates 1 day before transfection by the standard calcium phosphate precipitation method. Twenty-four hours after transfection, cells were fixed in a methanol/acetone [1:1 (v/v)] mixture and processed for indirect immunofluorescence staining as described previously (Chen and Chen, 2003). Antibodies used against HA-tag and SMRT were from Santa Cruz (Santa Cruz, CA) and GeneTex (San Antonio, TX), respectively. After extensive

washing, fluorescein isothiocyanate-conjugated goat anti-rabbit and rhodamine-conjugated goat anti-mouse antibodies were added directly to the coverslips. Cell nuclei were costained with 4',6-diamidino-2-phenylindole dihydrochloride hydrate (Sigma Chemical Co.). Coverslips were mounted with a ProLong Antifade kit (Invitrogen, Carlsbad, CA). Standard epifluorescence microscopy was performed on an inverted epifluorescence microscope (Axiovert 200; Zeiss, Thornwood, NY). The images were taken with a cool charge-coupled device camera (AxioCam) and analyzed by the Axiovision software (Zeiss).

Cell Culture and Transient Transfections. Human embryonic kidney 293 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and 5 μ g/ml gentamicin (Invitrogen). Cells (2×10^5) were seeded in 12-well plates and transfected by the standard calcium phosphate method. The amounts of each DNA used in the transfection were (unless otherwise stated): 1 μ g of reporter plasmid, 0.5 μ g of hPXR expression vector or other nuclear receptor, 0.5 μ g of SMRT or other cotransfected expression vector, and 0.5 μ g of the internal control pCMX- β -galactosidase; the total amount of DNA was brought up to 2.5 μ g per well by pGEM7. Transfections proceeded overnight, and precipitates removed by washing cells with phosphate-buffered saline supplemented with calcium and magnesium. Cells were cultured for an additional 48 h, after which the cells were harvested and analyzed for luciferase activity and β -galactosidase activities as described previously (Chen et al., 1996).

HepG2 cells were maintained in phenol red-free DMEM containing 10% FBS and antibiotic/antimycotic (Invitrogen). HepG2 cells (5×10^5) were seeded in six-well plates and transfected with FuGENE-6 (Roche Diagnostic, Indianapolis, IN). Transfection with pSG5-PXR or empty vector, pCMX-hSMRTe, CYP3A4-luciferase gene promoter construct, β -galactosidase, and pGEM7 was allowed to proceed for 4 h in serum-reduced Opti-MEM (Invitrogen). The transfection media was then removed and replaced with phenol red-free DMEM containing 10% charcoal-stripped FBS. Cells were then treated with vehicle control (DMSO) or 10 μ M Rif and incubated for an additional 48 h. Cells were then harvested and analyzed for luciferase and β -galactosidase activities.

The effect of SMRT siRNA on PXR transcriptional activity was analyzed in HEK293 and HepG2 cells. The SMRT and a negative control siRNAs were expressed from the pLL3.7 lentiviral vector (Rubinson et al., 2003). The targeted sequences for the SMRT siRNA is GGGTATCATCACCGCTGTG starting at nucleotide 5778 and amino acid 1875 of SMRT (Ishizuka and Lazar, 2003), and GGTC-CGGCTCCCCCAAATG for the negative control siRNA that has no homologous sequences in the vertebrate transcriptome.

Results

SMRT Interacts with PXR and Other Orphan Nuclear Receptors. The full-length hPXR contains 434 amino acids bearing 86% similarity with the mPXR (Kliewer et al., 1998). The SMRT sequence corresponds to that in hSMRTe (Park et al., 1999). The deletion and site-directed mutants of hPXR and SMRT used in this study are summarized in Fig. 1. We first tested the binding of hPXR and mPXR to GST-cSMRT by GST pull-down assay (Fig. 2). As expected, both hPXR and mPXR formed a stable complex with cSMRT. Likewise, we observed strong bindings of cSMRT with orphan nuclear receptors hLXR β (NR1H2) (Hu et al., 2003), mCOUP-TF1 (NR2F1), and mCOUP-TF2 (NR2F2) (Shibata et al., 1997; Bailey et al., 1998), v-erbA (Chen and Evans, 1995), and weakly with Rev-erbA α (NR1D1) (Downes et al., 1996) and mCAR. The cSMRT also bound strongly to the human farnesoid X-activated receptor (hFXR, NR1H4), but not the rat hepatocyte nuclear factor 4 (rHNF4), the *Drosophila melanogaster* ultraspiracle, or the hCAR. These data

show that SMRT interacts with PXR and several orphan nuclear receptors.

PXR Binds to the ID2 Domain of SMRT. To investigate how SMRT interacts with PXR, we analyzed the interacting surface using SMRT deletion and site-directed mutants. Both hPXR and mPXR bound strongly to the SMRT ID2 fragment but not to the ID1 fragment (Fig. 3A). Likewise, mPPAR γ and hLXR β bound to the ID2 fragment preferentially, whereas hRAR α preferred ID1. These results show that hRAR α prefers SMRT ID1, whereas PXR, PPAR γ , and LXR β interact with the ID2 of SMRT.

The contribution of each corepressor helix on SMRT-PXR interaction was further analyzed using site-directed mutants mID1, mID2, and mID1/2 in the context of cSMRT (Fig. 3B). Again, both hPXR and mPXR strongly bound to cSMRT and ID2 instead of ID1. The mID2 mutation consistently weakened the interaction more profoundly than the mID1 mutation. Although the mID1 mutation also had an effect on PXR binding with cSMRT, this might be caused by allosteric effect on the ID2-PXR interaction or a conformational change of cSMRT. The double mID1/2 mutation did not completely abolish PXR binding with cSMRT, suggesting that there

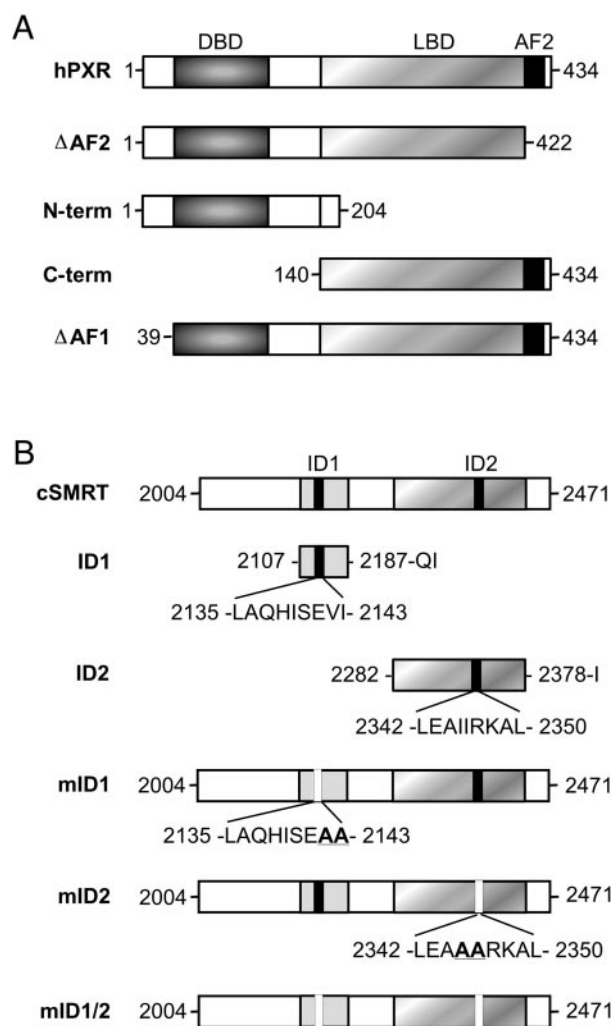


Fig. 1. Schematic of hPXR (A) and cSMRT (B) constructs. N-term, N-terminal fragment; C-term, C-terminal fragment; cSMRT, C-terminal domain of SMRT. The wild-type and mutated corepressor motif sequences are shown in ID1, ID2, mID1, and mID2. The mID1/2 contains both the mID1 and mID2 mutations.

could be additional PXR binding sites on SMRT. In contrast to PXR, the mID1 and mID1/2 mutations completely abolished hRAR α binding with cSMRT, whereas the mID2 and mID1/2 mutations completely abolished hLXR β binding (Fig. 3B, right). In both cases, mutation of the nonbinding ID (i.e., mID2 for hRAR α or mID1 for hLXR β) had little or no effect. These data indicate that SMRT uses the ID2 helix in interaction with PXR.

We next determined the region within PXR that are responsible for interacting with cSMRT. Three hPXR deletion mutants were generated and tested for their bindings with cSMRT, SMRT-ID1, and SMRT-ID2 fragments (Fig. 3C). We found that the Δ AF2 bound better to the cSMRT and ID2 fragments than to the wild-type hPXR. The separated N- and C-terminal fragments showed poor binding to cSMRT, ID1, or ID2 in this assay. In contrast, deletion of the N-terminal 39 amino acids from hPXR (Δ AF1) had little effect on cSMRT or ID2 binding. These data suggest that both the LBD and DBD of hPXR are essential for strong binding with SMRT in vitro, whereas the AF2 helix and AF1 region are not essential.

Effects of Ligands on PXR-SMRT Interaction in Vitro and in Yeast Cells. Most nuclear receptors undergo conformational changes to cause release of corepressors and recruitment of coactivators upon ligand binding. Therefore,

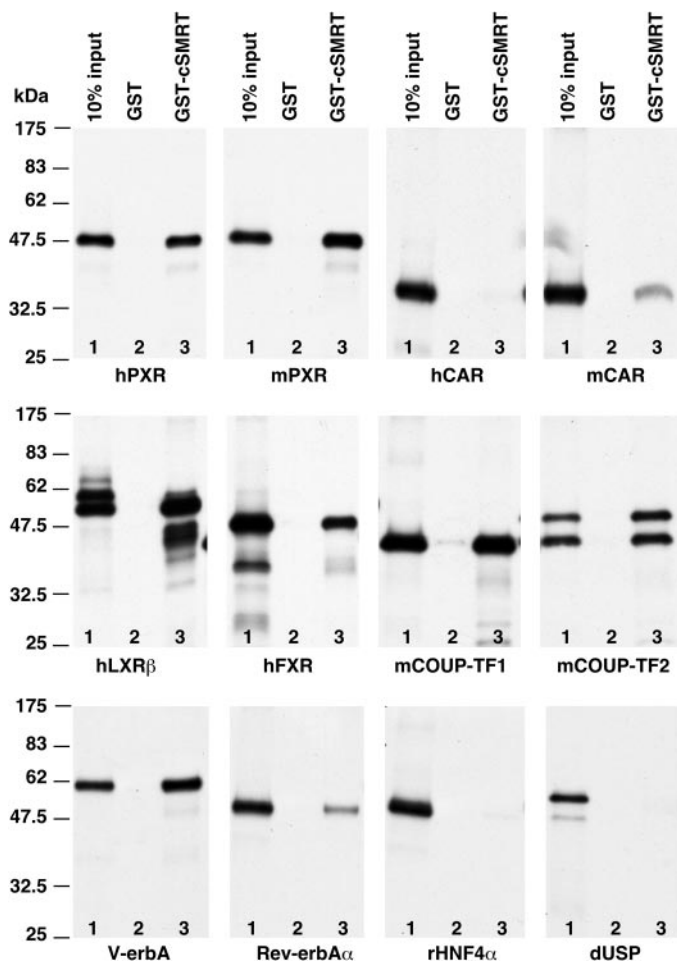


Fig. 2. SMRT interacts with orphan nuclear receptors. GST pull-down assays using GST-cSMRT and 35 S-labeled orphan receptors are shown. GST alone was used as control for background binding. h, human; m, mouse.

the effects of PXR ligands on PXR-SMRT interaction were examined. Increasing concentrations of Rif on hPXR binding to GST-cSMRT were examined by GST pull-down assay. We observed a Rif dose-dependent dissociation of cSMRT at 10 μ M and higher concentrations (Fig. 4A). This dissociation of hPXR-cSMRT complex was specific because Rif at all concentrations tested had no effect on hLXR β -cSMRT interaction. Similar results were observed using the ID2 fragment of SMRT (Fig. 4B), suggesting that Rif binding is indeed able to disrupt PXR-SMRT interaction in vitro.

We further analyzed the PXR-SMRT interaction and the effects of ligands by yeast two-hybrid assay. Consistent with the in vitro data, hPXR interacted strongly with SMRT in the absence of ligand (Fig. 5A). Compared with SMRT, hPXR did not interact with hNCoR, consistent with a previous report (Takeshita et al., 2002). Rif at 10 μ M caused a significant dissociation (approximately 2-fold) of the PXR-SMRT complex in this assay ($p < 0.01$). In contrast, Rif enhanced the PXR-RAC3 association by approximately 3.3-fold ($p < 0.01$). These data suggest that the unliganded PXR preferentially interacts with SMRT, whereas liganded PXR would prefer RAC3.

The ability of Rif to dissociate PXR-SMRT complex in the yeast two-hybrid assay was further analyzed in greater details by measuring the effects of Rif over a concentration range of 5 orders of magnitude. As expected, Rif diminished the PXR-SMRT interaction and increased the PXR-RAC3

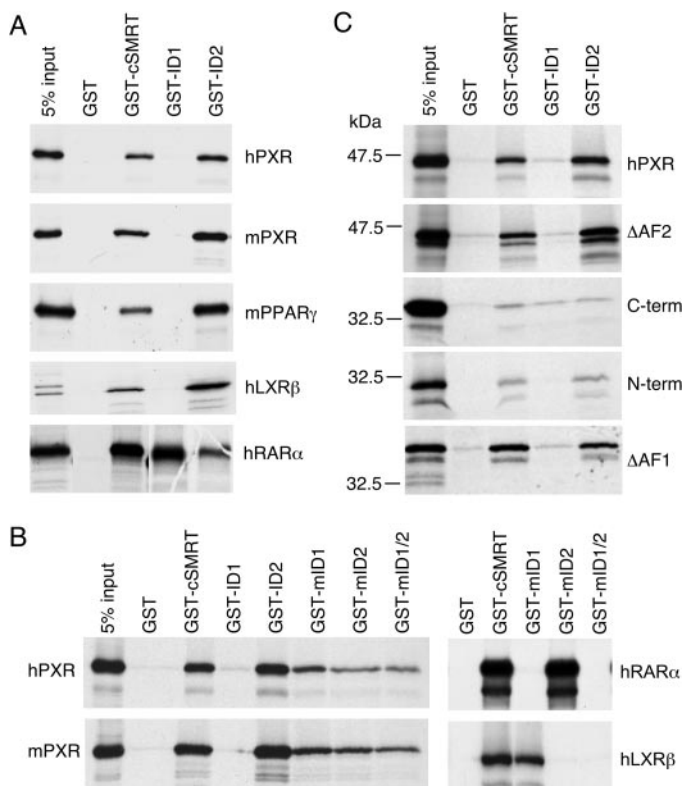


Fig. 3. PXR binds preferentially to SMRT-ID2. A, GST pull-down assay showing bindings of 35 S-labeled hPXR, mPXR, mPPAR γ , hLXR β , and hRAR α to GST, GST-cSMRT, GST-ID1, and GST-ID2. PXR α , mPPAR γ , and hLXR β bound to cSMRT and ID2 but not ID1. In contrast, hRAR α preferentially bound to ID1. B, bindings of hPXR, mPXR, hRAR α , and hLXR β to cSMRT, ID1, ID2, mID1, mID2, and mID1/2 mutants of SMRT in GST pull-down assay. C, relative bindings of various PXR fragments with cSMRT, SMRT-ID1, and SMRT-ID2.

interaction in a concentration-dependent manner (Fig. 5B). When the Rif concentration was equal to or below 1 μM , hPXR preferentially interacted with SMRT, whereas PXR bound more strongly to RAC3 at concentrations of Rif ≥ 10 μM . We estimated that Rif would cause an exchange of corepressor to coactivator at approximately 7 μM in this assay. This concentration of Rif corresponds well to the EC_{50} that produced 50% of the maximal SMRT dissociation and RAC3 association with hPXR.

It is known that different ligands exert species-specific effects on PXR activation. Therefore, we tested the effects of various known PXR ligands on hPXR-SMRT interaction in yeast two-hybrid assay (Fig. 5C). Consistent with previous data, Rif at 10 μM caused an approximately 2.3-fold dissociation of the hPXR-SMRT complex ($P < 0.01$). The human PXR-specific ligand CTZ caused an even greater dissociation (13.7-fold) of the hPXR-SMRT complex. In contrast, the mouse PXR-specific ligands PCN and DEX had no significant

effect on the hPXR-SMRT interaction ($P > 0.1$). These data confirm the species-specific effects of PXR ligands on disrupting PXR-SMRT interaction in yeast cells.

The PXR fragments responsible for SMRT interaction and the effects of Rif on the interaction were then determined by the same assay (Fig. 5D). Again, Rif caused a consistent and significant 3-fold dissociation of the wild-type PXR-SMRT complex ($p < 0.01$). The ΔAF2 and C-terminal fragments interacted with cSMRT approximately 40 to 50% stronger than the full-length hPXR ($p < 0.01$). Furthermore, Rif caused a significant dissociation of the C-terminal-SMRT interaction ($p < 0.01$), but it had no effect on the ΔAF2 -SMRT interaction. The N-terminal fragment showed no interaction with cSMRT in this assay. These data suggest that the PXR-SMRT interaction is sensitive to Rif in yeast cells, that PXR interacts with SMRT through the C-terminal domain, and that the AF2 helix of PXR inhibits SMRT binding and is essential for Rif-dependent dissociation of SMRT.

PXR Colocalizes with SMRT in Mammalian Cells.

SMRT is localized in the nuclei of mammalian cells in a speckle pattern (Park et al., 1999; Downes et al., 2000; Wu et al., 2001). To further demonstrate the PXR-SMRT interaction, we tested colocalization of cotransfected hPXR and SMRT by double immunofluorescence staining (Fig. 6). When expressed alone, hPXR displays diffuse nuclear staining similar to that of many nuclear receptors (Kawana et al., 2003). It is noteworthy that when coexpressed with SMRT, the wild type, ΔAF2 , and C-terminal hPXR colocalized with SMRT at many nuclear foci (Fig. 6), indicating an association in the cultured mammalian cells. The N-terminal hPXR and hCAR showed no evidence of colocalization with SMRT. These data suggest that hPXR could interact with SMRT and be recruited to SMRT nuclear foci in mammalian cells.

SMRT Inhibits PXR Transcriptional Activity. To determine whether PXR regulates transcription in the absence of ligand, we analyzed its intrinsic transcriptional activity using Gal4 DBD-PXR fusion proteins. The full-length and various PXR domains were fused with Gal4 DBD, and the effects on a Gal4-dependent luciferase reporter were analyzed in HEK293 cells by transient transfection. As shown in Fig. 7A, the full-length PXR and all PXR fragments displayed significant repression activity in comparison with Gal4 DBD alone ($p < 0.01$), although differences among various PXR fragments were less significant. We observed ~ 7.9 -fold repression by the full-length hPXR, 10.2-fold repression by ΔAF2 , and 4.8- and 3.7-fold repression by C- and N-terminal fragments, respectively. The stronger repression observed with ΔAF2 correlates with its stronger interaction with SMRT (Fig. 5D), suggesting that repression by PXR is in part mediated by SMRT. In contrast, repression by the PXR N-terminal fragment could be due to interactions with other corepressors because the N-terminal fragment does not interact with SMRT. Rif partially reversed the repression mediated by full-length hPXR and C-terminal fragment by approximately 2.1-fold ($p < 0.05$) and 2.8-fold ($p < 0.01$), respectively, whereas Rif had no effect on ΔAF2 and N-terminal fragment, as expected, in that they do not contain a ligand binding domain. These data strongly suggest that hPXR contains multiple repression domains, and Rif could partially release the repression activity mediated by PXR LBD domain.

Next, we examined the PXR-SMRT interaction in COS-7

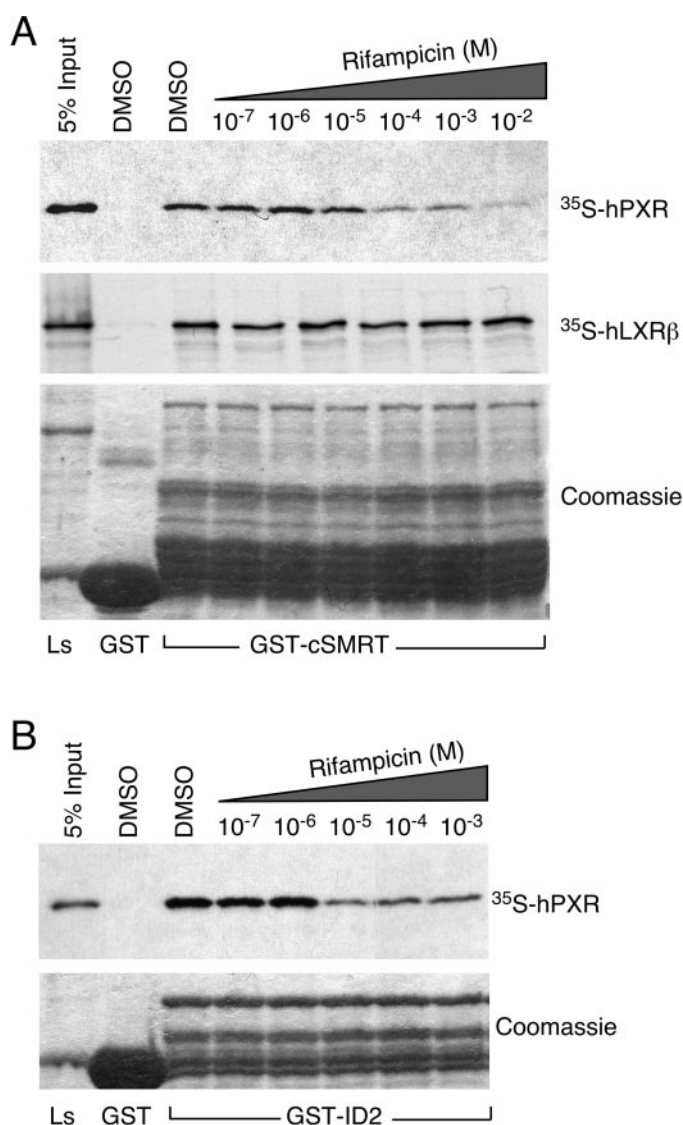


Fig. 4. Rifampicin blocks PXR-SMRT interaction. A, rifampicin inhibited hPXR binding to GST-cSMRT in a concentration-dependent manner. As control, rifampicin had no effect on hLXR β binding to GST-cSMRT. Ls, rabbit reticulocyte lysate. B, rifampicin inhibited hPXR binding to GST-SMRT-ID2 in a concentration-dependent manner.

cells by mammalian two-hybrid assay (Fig. 7B). Without ligand, VP16-hPXR interacted with Ga4-cSMRT by 2.3-fold ($p < 0.05$). Deletion of AF2 helix from PXR enhanced the PXR-SMRT interaction by another 2.4-fold ($p < 0.01$), suggesting that Δ AF2 interacts with SMRT more strongly than wild-type PXR. Addition of 10 μ M Rif disrupted the PXR-SMRT association by 2.9-fold ($p < 0.05$), whereas Rif had no significant effect on the SMRT- Δ AF2 interaction ($P > 0.05$ in

ANOVA and Tukey HSD test). As control, cSMRT interacted with hRAR α strongly (4.6-fold), and *all-trans* retinoic acid disrupted the interaction completely. These results support our hypothesis that PXR could interact with SMRT in mammalian cells, that such an interaction is sensitive to Rif treatment, and that deletion of the AF2 helix enhanced PXR-SMRT interaction.

The functional consequence of PXR-SMRT interaction on

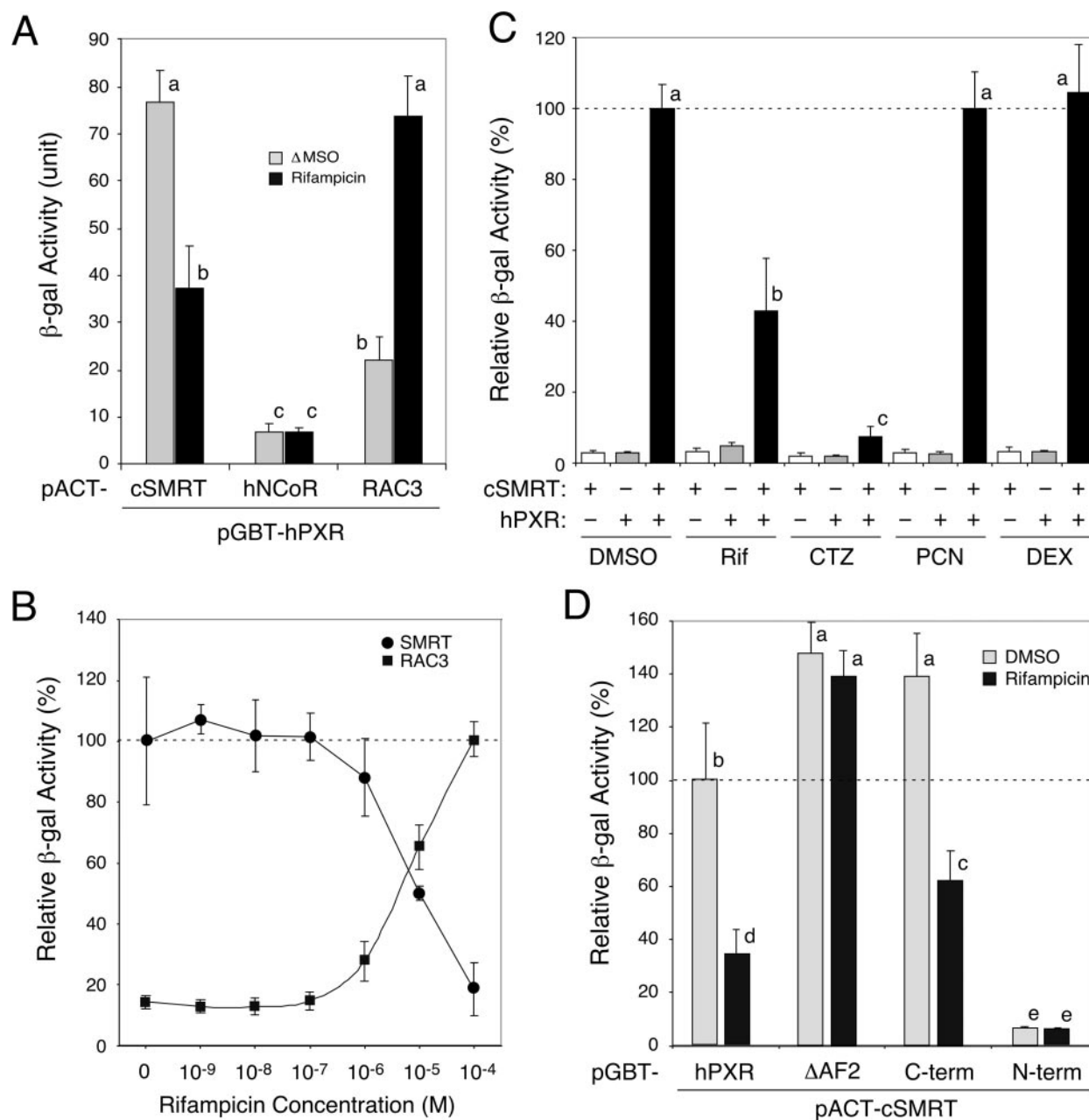


Fig. 5. Interaction between PXR and SMRT in yeast two-hybrid assay. A, hPXR interacts with SMRT and RAC3 in yeast two-hybrid assay. Full-length hPXR was expressed as Gal4 DBD fusion in the pGBT9 vector (BD Biosciences). cSMRT, hNCoR (aa 2228–2440), and RAC3 (aa 1–1204) were expressed as Gal4 AD fusions in pACT2 or pGAD424 (RAC3) vectors. β -Galactosidase activities were determined from three independent cultures treated with DMSO (solvent) or Rif (10 μ M) for 36 h. B, Rif causes an exchange of SMRT to RAC3 in hPXR binding in yeast two-hybrid assay. Yeast colonies cotransformed with pGBT-hPXR and pACT-cSMRT or pGAD-RAC3 (1–1204) were analyzed for β -galactosidase expression after treatments with increasing concentrations of Rif. The exchange of cofactor binding occurs at approximately 7 μ M Rif. C, Rif and CTZ disrupt hPXR-cSMRT interaction. Yeast cells cotransformed with pGBT-hPXR and pACT-cSMRT were treated with hPXR-specific ligands Rif or CTZ, and mPXR-specific ligands PCN or DEX (10 μ M each) for 48 h. β -Galactosidase activities were determined from three independent transformants. Both Rif and CTZ disrupted hPXR-cSMRT interaction, but PCN and DEX had no effect. D, PXR fragments that interact with SMRT in yeast. Yeast colonies containing pACT-cSMRT and pGBT-hPXR, Δ AF2, C-term fragment, or N-term fragment were analyzed for β -galactosidase expression after solvent (DMSO) or Rif (10 μ M) treatment. Statistical analyses were conducted via one-way ANOVA and Tukey HSD test. Samples were grouped into statistically significantly different categories as labeled on top of each column.

transcriptional activity of hPXR was determined by transient transfection using a human primary hepatoma cell line HepG2. HepG2 cells were transfected with full-length hPXR, full-length SMRT, and a 13-kilobase CYP3A4 promoter-driven luciferase reporter (Goodwin et al., 1999). In the absence of ligand, overexpression of hPXR alone activated the CYP3A4 promoter activity by approximately 6.6-fold ($p < 0.01$; Fig. 7C). Similar levels of PXR-dependent activation were observed previously (Takeshita et al., 2002). This effect may be due to endogenous PXR activators. Transfection of SMRT alone had a slight but nonsignificant effect on the CYP3A4 promoter activity. However, cotransfection of SMRT significantly attenuated the hPXR-mediated activation of the CYP3A4 promoter by 51% ($p < 0.01$). These results suggest that SMRT could potentially inhibit hPXR-mediated transactivation of the CYP3A4 promoter in cultured hepatic cells.

To further support a role of SMRT in regulating CYP3A4 expression, we silenced SMRT expression in HEK293 cells by shRNA and analyzed the CYP3A4 promoter activity (Fig. 7D). As expected, Rif significantly activated the reporter gene (3.8-fold, $p < 0.01$), and the control shRNA had no effect on CYP3A4 activation ($p = 0.15$). In contrast, the SMRT shRNA enhanced Rif-activated reporter activity by 4.7-fold ($p < 0.01$). Western blot analysis showed that the control shRNA had no effect on SMRT silencing, whereas the SMRT shRNA blocked the expression of a cotransfected SMRT fragment (aa

475–981) (Fig. 7D, inset). These data suggest that the effects of SMRT shRNA are caused by silencing of endogenous SMRT expression and that SMRT silencing may lead to enhancement of PXR-stimulation of CYP3A4 promoter in hepatic cells.

Discussion

In this study, we investigated the physical and functional interactions between the orphan nuclear receptor PXR and the nuclear corepressor SMRT. We found that SMRT uses the ID2 domain to interact with the LBD of PXR. The interaction between PXR and SMRT could be disrupted by PXR ligands such as Rif. The AF2 helix of PXR seems to be inhibitory to SMRT binding. We also found that hPXR could be recruited by SMRT to discrete nuclear foci and showed that SMRT is inhibitory to the transcriptional activity of PXR. Taken together, these data provide strong evidence that SMRT plays a role in regulating the transcriptional activity of the orphan nuclear receptor PXR.

Using a GST pull-down assay, a direct interaction between PXR and SMRT was first observed in this study. This finding is consistent with previous reports using mammalian two-hybrid assays (Synold et al., 2001; Takeshita et al., 2002). In addition to PXR, SMRT also interacts with LXR β and farnesoid X receptor but not with CAR. The binding with LXR β is

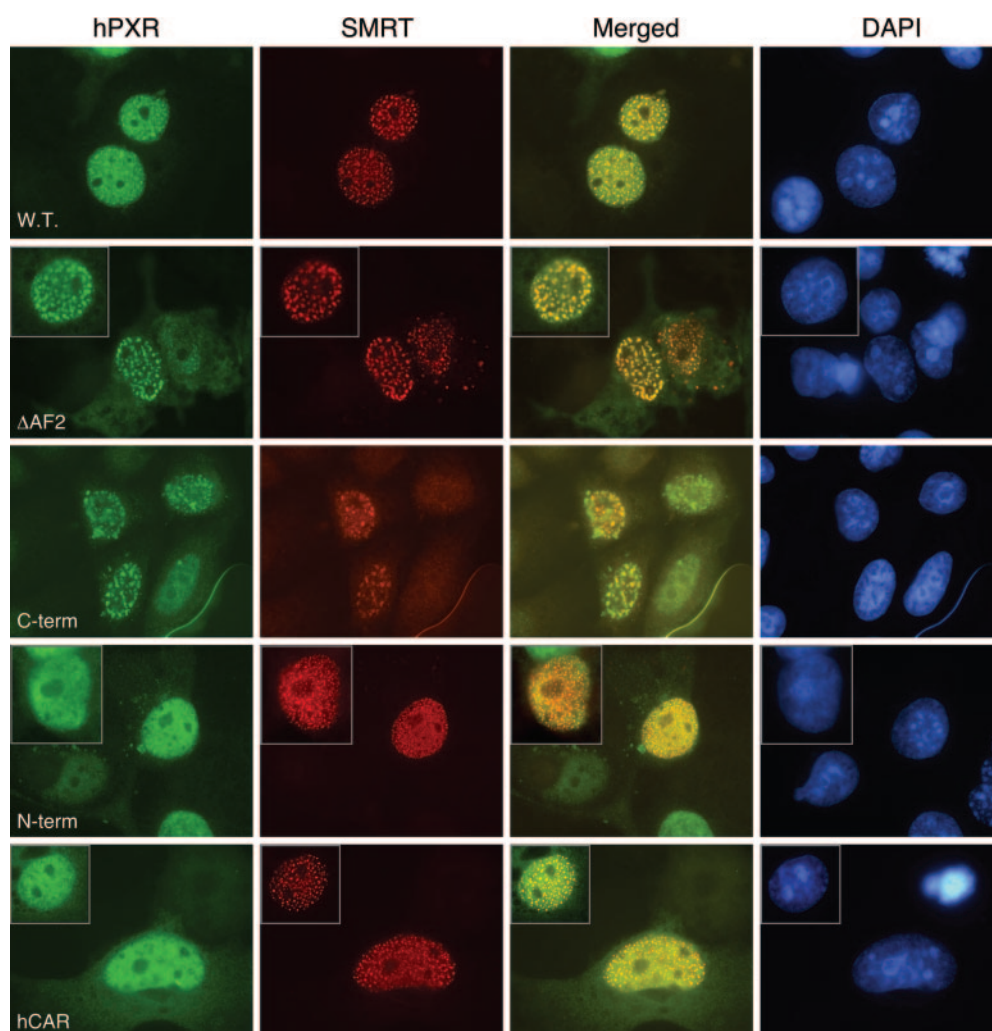


Fig. 6. PXR colocalizes with SMRT in mammalian cells. COS-7 cells were cotransfected with human SMRT and HA-hPXR, or indicated hPXR constructs including Δ AF2, C-terminal, N-terminal, and HA-hCAR. SMRT was detected with a mouse anti-SMRT monoclonal antibody (Gene-Tex, Inc.), followed by a rhodamine-conjugated goat anti-mouse secondary antibody. PXR and hCAR were detected by a rabbit anti-HA polyclonal antibody (Santa Cruz, Inc.), followed by a fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody. Cell nuclei were costained with 4',6-diamidino-2-phenylindole dihydrochloride hydrate. The wild-type (W.T.) hPXR and its Δ AF2 and C-terminal fragments show colocalization with SMRT at nuclear foci, whereas the hPXR N-terminal and hCAR were not recruited to the SMRT nuclear foci. The boxed cells were from different fields in the same slide.

consistent with a previous report (Hu et al., 2003). SMRT binds promiscuously to several nuclear receptors, using either the ID1 or ID2 domain (Li et al., 1997; Cohen et al., 2000; Ghosh et al., 2002). We found that PXR and LXR β both prefer ID2 (Fig. 3A). The mechanism for such a preference is likely to involve molecular recognitions between the cofactor-binding pocket on the LBD of the receptor and specific configuration of the ID2 helix. Because disruption of the ID2 helix did not completely block PXR binding (Fig. 3A), we speculate that SMRT may contain additional binding surfaces for PXR and/or the mechanism of SMRT-PXR interaction may be different from other nuclear receptors.

The corepressor helix is known to bind to a site in the LBD of the receptor that overlaps with the coactivator-binding pocket formed by helices 3, 4, and 5 (Shiau et al., 1998; Gampe et al., 2000). The AF2 helix of the receptor could interfere with the corepressor binding because of its ability to

adopt an "active" conformation, which blocks corepressor binding. Similar to other nuclear receptors, deletion of the AF2 helix stabilizes PXR-SMRT interaction. Truncation of hPXR into the C-terminal LBD and N-terminal DBD fragments seems to diminish SMRT binding in the GST pull-down assay (Fig. 3C), whereas strong interaction between the C-terminal LBD and SMRT could be observed in yeast (Fig. 5D) and by colocalization study (Fig. 6). It is possible that a proper intramolecular folding of the LBD in reticulocyte lysate requires the DBD, whereas chaperon proteins present in yeast and mammalian cells might compensate for such a requirement. The heterologous Gal4 DBD used in the two-hybrid assays might also compensate for such a requirement.

The effect of PXR ligands on the PXR-SMRT interaction was also investigated. Activation of a nuclear receptor by its agonist ligand usually causes a release of corepressors, and

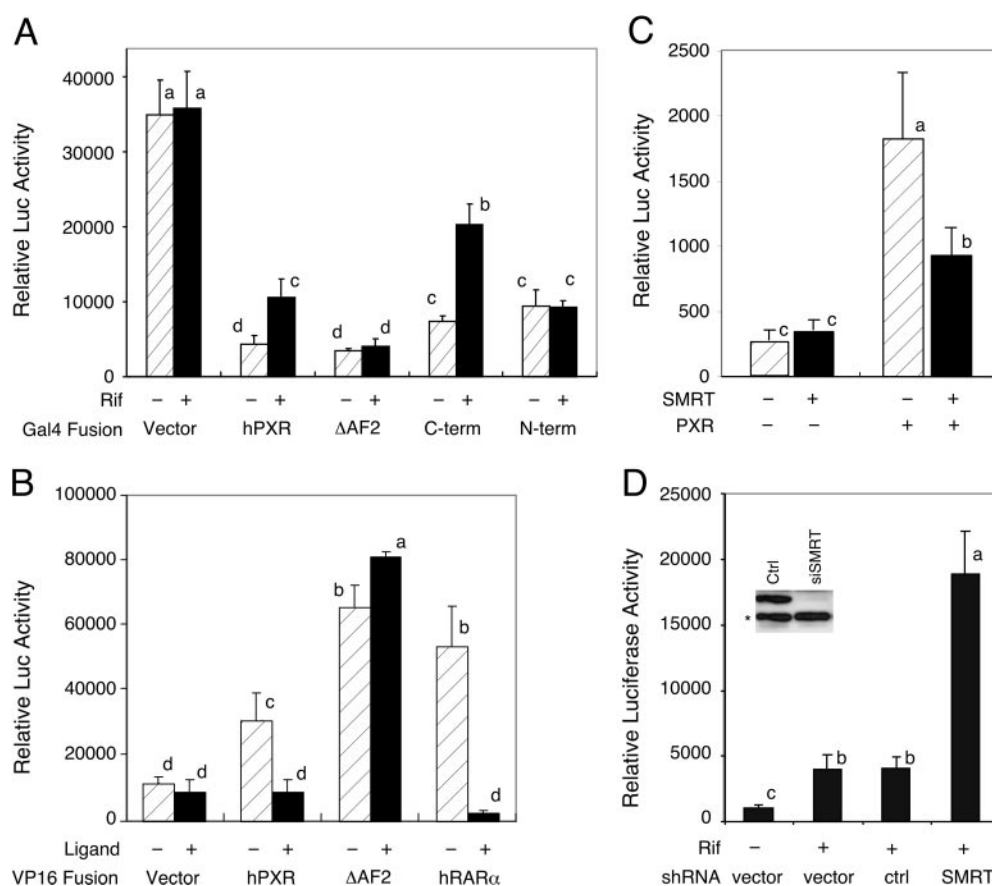


Fig. 7. Regulation of PXR activity by SMRT. A, Gal4 DBD-hPXR fusions repressed basal transcription. HEK293 cells were transfected with Gal4 DBD vector, Gal4 DBD fusions of hPXR, Δ AF2, C-terminal, or N-terminal fragment, together with the Gal4-dependent MH100-tk-luc reporter and a β -galactosidase expression vector as internal control. Transfected cells were treated with Rif (10 μ M) or solvent (DMSO) for 48 h. The -fold repression was shown on top of each column as a negative value. The fold activation is also indicated in parentheses on top of each column. B, mammalian two-hybrid assay showing interactions of Gal4 DBD-cSMRT with VP16-hPXR and VP16- Δ AF2 mutant. HEK293 cells were transfected with Gal4 DBD-cSMRT and indicated VP16 AD fusions, along with the Gal4-dependent MH100-tk-luc reporter and a β -galactosidase expression vector. After transfection, cells were refed with fresh media containing Rif (10 μ M for the vector, hPXR, and Δ AF2) or *all-trans* retinoic acid (1 μ M for hRAR α), or solvent (DMSO) alone as indicated. C, SMRT inhibits hPXR basal activity on CYP3A4 promoter in HepG2 cells. Cells were transfected with hPXR or empty vector with or without SMRT together with a CYP3A4-luciferase reporter and β -galactosidase control. Relative luciferase activities were determined from three independent experiments. Cotransfection of PXR stimulated CYP3A4 promoter activity by 6.6-fold, whereas coexpression of SMRT inhibits the PXR-dependent activation by 51% ($p < 0.01$). D, silencing of SMRT enhances PXR transcriptional activity. HEK293 cells were mock-transfected (-) or were transfected with pLL3.7 vector (vector), a scramble control shRNA (ctrl), or the SMRT shRNA (SMRT) expression vector, along with the hPXR and CYP3A4-Luc reporter. Relative luciferase activities were determined from three independent transfections in the absence (-) or presence (+) of Rif (10 μ M). The inset shows that the SMRT shRNA blocked the expression of a cotransfected HA-tagged SMRT fragment (aa 475-981) that contains the RNAi target site. *, nonspecific band detected by Western blot with anti-HA antibody. The samples were grouped into statistically significantly different categories as labeled on top of each column. The difference between c and d groups in A are significant in LSD test, but nonsignificant in Tukey HSD test at 0.05 level. The difference between the two group samples in B are not significant in Tukey HSD test but significant in LSD test at 0.05 level.

allows recruitments of coactivators. It is ironic that a prior report using mammalian two-hybrid assays showed that Rif enhanced the PXR-SMRT interaction by approximately 2-fold (Takeshita et al., 2002). In contrast, we found that Rif causes dissociation of the PXR-SMRT complex in multiple assays. This finding is in agreement with an earlier result showing that paclitaxel disrupts PXR-SMRT interaction (Synold et al., 2001). We also found that the Rif-dependent dissociation of PXR-SMRT complex requires the AF2 helix of PXR, implicating a prerequisite conformational change of the AF2 helix before the release of corepressor. The EC₅₀ of Rif required for 50% dissociation of SMRT and 50% association of RAC3 was approximately 7 μ M, similar to the concentration required for activation of PXR in mammalian cells (Lehmann et al., 1998; Xie et al., 2000b). We noticed that, in the mammalian two-hybrid assay, Takeshita et al. (2002) used the Gal4 DBD-PXR fusion, which could be activated by Rif. Such activation might have obscured the interpretation of their two-hybrid data. Both our study and the study by Synold et al. (2001) used the VP16-hPXR fusion, which could not activate the reporter. In addition to the Rif-induced dissociation of PXR-SMRT complex, the anticipated species-specific effects were also observed with hPXR and mPXR ligands in our yeast assay (Fig. 5C). These results strongly suggest that Rif disrupts PXR-SMRT interaction, resulting in release of corepressors and recruitment of coactivators.

The association of PXR and SMRT in mammalian cells was also demonstrated by immunofluorescence colocalization study (Fig. 6). Such colocalization reflects an interaction of the two proteins in mammalian cells. Both SMRT and PXR are known to shuttle between nucleus and cytoplasm under various conditions (Hong and Privalsky, 2000; Zelko et al., 2001; Kawana et al., 2003). Although recent studies have shown that unliganded endogenous mPXR may be located in the cytoplasm of mouse liver (Kawana et al., 2003; Squires et al., 2004), it is clear that when expressed in mammalian cells, PXR is localized in a diffuse pattern in the nucleus (Zelko et al., 2001; Kawana et al., 2003). SMRT is concentrated at discrete nuclear foci when expressed in culture cells (Park et al., 1999; Hong and Privalsky, 2000; Wu et al., 2001). Coexpression of SMRT and PXR resulted in recruitment of PXR into the SMRT nuclear foci (Fig. 6). This effect was most apparent with the Δ AF2 mutant and the C-terminal fragment of hPXR. The recruitment of hPXR to SMRT nuclear foci seems to rely on interaction between PXR and SMRT. The function of the SMRT nuclear foci is currently unknown, although a prior report suggested that they could be sites of deacetylation (Downes et al., 2000). The accumulation of PXR to the SMRT nuclear foci might then serve a function to repress gene expression by tethering target promoters to the foci. Then again, PXR might be a substrate of the deacetylase, or that SMRT nuclear foci might be storage sites for PXR and other nuclear receptors.

The above data suggest that PXR might repress basal transcription in the absence of ligand. Indeed, Gal4 DBD-hPXR repressed basal promoter activity of a Gal4-dependent reporter (Fig. 7A). We found that removal of the AF2 helix slightly enhanced such a repressor activity of hPXR, correlating with an enhanced interaction with SMRT. Likewise, the C-terminal fragment also repressed basal transcription and interacted with SMRT. Both the full-length and C-terminal fragment of hPXR were able to stimulate transcription

in response to Rif, correlating with the ability of Rif to dissociate their interactions with the corepressor. In contrast, the Δ AF2 mutant could not respond to Rif because Rif could not cause dissociation with SMRT. Finally, we speculate that the repressor activity of the N-terminal fragment of hPXR might be due to interaction with other unknown corepressors.

A previous PXR knockout mice study demonstrated that the *Cyp3a11* mRNA in the liver of the knockout mice was increased by \sim 4-fold compared with wild-type mice (Staudinger et al., 2001), although another independent PXR knockout mice study concluded that loss of PXR did not alter the basal expression of *Cyp3a* (Xie et al., 2000a). Although the basis for this difference is unknown, the data presented by Xie et al. (2000a) seem to show a slight increase of *Cyp3a11* basal expression in the liver of the PXR-null mice (Fig. 1C in that study). It is possible that such a difference might arise from variations among different mouse lines, and PXR might differentially regulate basal expression of its target genes in different tissues. We further analyzed the effect of SMRT on PXR-regulated human *Cyp3a4* promoter activity in hepatic HepG2 cells. Transfection of hPXR enhanced the reporter activity by approximately 6.6-fold (Fig. 7C). The reporter we used contains a 13-kilobase promoter/enhancer element, thus its regulation must be under stringent control by other transcription factors, such as the CCAAT enhancer-binding protein and hepatocyte nuclear factor-3, as described in a prior study (Rodriguez-Antona et al., 2003). On the other hand, our stripped serum or culture media, or the HepG2 cells, might contain endogenous PXR ligands, because PXR is known to be promiscuous on the selection of ligands. Nonetheless, under this low level of activation, SMRT was able to suppress the activation of the *Cyp3a4* promoter by approximately 50% (Fig. 7C). In addition, silencing of SMRT expression by siRNA greatly enhanced *Cyp3a4* promoter activity (Fig. 7D). Taken together, these results suggest that PXR preferentially binds to the corepressor SMRT in the absence of low levels of ligand and that SMRT may inhibit the transcriptional activity of PXR and the expression of PXR target promoter. However, we note that these results are limited to cultured mammalian cells and may not reflect what occurs with the *CYP3A4* promoter in animal. Further investigations are thus needed to establish a definitely role of SMRT in regulating *CYP3A4* gene expression in vivo.

Acknowledgments

We are grateful to Steve Kliewer for providing the PXR expression vectors, Christopher Liddle for the *CYP3A4*-luc reporter, and Shou-En Lu for help in statistical analyses.

References

- Bailey P, Sartorelli V, Hamamori Y, and Muscat GE (1998) The orphan nuclear receptor, COUP-TF II, inhibits myogenesis by post-transcriptional regulation of MyoD function: COUP-TF II directly interacts with p300 and myoD. *Nucleic Acids Res* 26:5501–5510.
- Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeborg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P, and Berkenstam A (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci USA* 95:12208–12213.
- Blumberg B, Sabbagh W Jr, Juguilon H, Bolado J Jr, van Meter CM, Ong ES, and Evans RM (1998) SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev* 12:3195–3205.
- Chen JD and Evans RM (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature (Lond)* 377:454–457.
- Chen JD, Umesono K, and Evans RM (1996) SMRT isoforms mediate repression and

- anti-repression of nuclear receptor heterodimers. *Proc Natl Acad Sci USA* **93**: 7567–7571.
- Chen LY and Chen JD (2003) Daxx silencing sensitizes cells to multiple apoptotic pathways. *Mol Cell Biol* **23**:7108–7121.
- Cohen RN, Putney A, Wondisford FE, and Hollenberg AN (2000) The nuclear corepressors recognize distinct nuclear receptor complexes. *Mol Endocrinol* **14**: 900–914.
- Downes M, Burke LJ, Bailey PJ, and Muscat GE (1996) Two receptor interaction domains in the corepressor, N-CoR/RIP13, are required for an efficient interaction with Rev-erbA alpha and RVR: physical association is dependent on the E region of the orphan receptors. *Nucleic Acids Res* **24**:4379–4386.
- Downes M, Ordentlich P, Kao HY, Alvarez JG, and Evans RM (2000) Identification of a nuclear domain with deacetylase activity. *Proc Natl Acad Sci USA* **97**:10330–10335.
- Gampe RT Jr, Montana VG, Lambert MH, Miller AB, Bledsoe RK, Milburn MV, Kliewer SA, Willson TM, and Xu HE (2000) Asymmetry in the PPARGgamma/RXRalpha crystal structure reveals the molecular basis of heterodimerization among nuclear receptors. *Mol Cell* **5**:545–555.
- Ghosh JC, Yang X, Zhang A, Lambert MH, Li H, Xu HE, and Chen JD (2002) Interactions that determine the assembly of a retinoid X receptor/corepressor complex. *Proc Natl Acad Sci USA* **99**:5842–5847.
- Goodwin B, Hodgson E, and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* **56**:1329–1339.
- Guengerich FP (1999) Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu Rev Pharmacol Toxicol* **39**:1–17.
- Hong SH and Privalsky ML (2000) The SMRT corepressor is regulated by a MEK-1 kinase pathway: inhibition of corepressor function is associated with SMRT phosphorylation and nuclear export. *Mol Cell Biol* **20**:6612–6625.
- Horlein AJ, Naar AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Soderstrom M, Glass CK, et al. (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature (Lond)* **377**:397–404.
- Hu X and Lazar MA (1999) The CoNR motif controls the recruitment of corepressors by nuclear hormone receptors. *Nature (Lond)* **402**:93–96.
- Hu X, Li S, Wu J, Xia C, and Lala DS (2003) Liver X receptors interact with corepressors to regulate gene expression. *Mol Endocrinol* **17**:1019–1026.
- Ishizuka T and Lazar MA (2003) The N-CoR/histone deacetylase 3 complex is required for repression by thyroid hormone receptor. *Mol Cell Biol* **23**:5122–5131.
- Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, et al. (2000) The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinol* **14**:27–39.
- Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, Tontonoz P, Kliewer S, Willson TM, and Edwards PA (2002) Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor and constitutive androstane receptor. *J Biol Chem* **277**:2908–2915.
- Kawana K, Ikuta T, Kobayashi Y, Gotoh O, Takeda K, and Kawajiri K (2003) Molecular mechanism of nuclear translocation of an orphan nuclear receptor, SXR. *Mol Pharmacol* **63**:524–531.
- Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, et al. (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92**:73–82.
- Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, and Kliewer SA (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* **102**:1016–1023.
- Li H, Leo C, Schroen DJ, and Chen JD (1997) Characterization of receptor interaction and transcriptional repression by the corepressor SMRT. *Mol Endocrinol* **11**:2025–2037.
- Ordentlich P, Downes M, and Evans RM (2001) Corepressors and nuclear hormone receptor function. *Curr Top Microbiol Immunol* **254**:101–116.
- Park EJ, Schroen DJ, Yang M, Li H, Li L, and Chen JD (1999) SMRTe, a silencing mediator for retinoid and thyroid hormone receptors- extended isoform that is more related to the nuclear receptor corepressor. *Proc Natl Acad Sci USA* **96**: 3519–3524.
- Ripp SL, Fitzpatrick JL, Peters JM, and Prough RA (2002) Induction of CYP3A expression by dehydroepiandrosterone: involvement of the pregnane X receptor. *Drug Metab Dispos* **30**:570–575.
- Rodriguez-Antona C, Bort R, Jover R, Tindberg N, Ingelman-Sundberg M, Gomez-Lechon MJ, and Castell JV (2003) Transcriptional regulation of human CYP3A4 basal expression by CCAAT enhancer-binding protein α and hepatocyte nuclear factor-3 γ . *Mol Pharmacol* **63**:1180–1189.
- Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Rooney DL, Ihrig MM, McManus MT, Gertler FB, et al. (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* **33**:401–406.
- Schuetz EG, Brimer C, and Schuetz JD (1998) Environmental xenobiotics and the antihormones cyproterone acetate and spironolactone use the nuclear hormone pregnenolone X receptor to activate the CYP3A23 hormone response element. *Mol Pharmacol* **54**:1113–1117.
- Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, and Greene GL (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**:927–937.
- Shibata H, Nawaz Z, Tsai SY, O'Malley BW, and Tsai MJ (1997) Gene silencing by chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI) is mediated by transcriptional corepressors, nuclear receptor-corepressor (N-CoR) and silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT). *Mol Endocrinol* **11**:714–724.
- Squires EJ, Sueyoshi T, and Negishi M (2004) Cytoplasmic localization of pregnane X receptor and ligand-dependent nuclear translocation in mouse liver. *J Biol Chem* **279**:49307–49314.
- Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, et al. (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci USA* **98**:3369–3374.
- Stedman CA, Liddle C, Coulter SA, Sonoda J, Alvarez JG, Moore DD, Evans RM, and Downes M (2005) Nuclear receptors constitutive androstane receptor and pregnane X receptor ameliorate cholestatic liver injury. *Proc Natl Acad Sci USA* **102**:2063–2068.
- Synold TW, Dussault I, and Forman BM (2001) The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. *Nat Med* **7**:584–590.
- Takeshita A, Taguchi M, Koibuchi N, and Ozawa Y (2002) Putative role of the orphan nuclear receptor SXR (steroid and xenobiotic receptor) in the mechanism of CYP3A4 inhibition by xenobiotics. *J Biol Chem* **277**:32453–32458.
- Wu X, Li H, Park EJ, and Chen JD (2001) SMRTe inhibits MEF2C transcriptional activation by targeting HDAC4 and 5 to nuclear domains. *J Biol Chem* **276**:24177–24185.
- Xie W, Barwick JL, Downes M, Blumberg B, Simon CM, Nelson MC, Neuschwander-Tetri BA, Brunt EM, Guzelian PS, and Evans RM (2000a) Humanized xenobiotic response in mice expressing nuclear receptor SXR. *Nature (Lond)* **406**:435–439.
- Xie W, Barwick JL, Simon CM, Pierce AM, Safe S, Blumberg B, Guzelian PS, and Evans RM (2000b) Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. *Genes Dev* **14**:3014–3023.
- Zelko I, Sueyoshi T, Kawamoto T, Moore R, and Negishi M (2001) The peptide near the C terminus regulates receptor CAR nuclear translocation induced by xenobiotics in mouse liver. *Mol Cell Biol* **21**:2838–2846.

Address correspondence to: Dr. J. Don Chen, Department of Pharmacology, University of Medicine and Dentistry of New Jersey, 661 Hoes Lane, Piscataway, NJ 08854. E-mail: chenjd@umdnj.edu
