Interaction of silencing mediator for retinoid and thyroid receptors with steroid and xenobiotic receptor on multidrug resistance 1 promoter.

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Abstract

Aims: The steroid and xenobiotic receptor (SXR) regulates the transcription of its target genes by interacting with various nuclear receptor cofactors. We have previously shown that silencing mediator for retinoid and thyroid receptors (SMRT) interacts with SXR even in the presence of rifampicin on cytochrome P450 monooxygenase 3A4 (*CYP3A4*) promoter in HepG2 cells. To examine the specificity of such interaction, the involvement of SMRT on SXR-mediated transcription through multidrug resistance (*MDR*) 1 gene promoter was examined using LS174T intestine-derived clonal cells.

Main methods: Transient transfection-based reporter gene assay was carried out to examine the effect of SMRT or nuclear receptor corepressor (NCoR) on SXR-mediated transcription in LS174T cells. Semi-quantitative RT-PCR was performed to confirm the expression of *MDR1* mRNA in LS174T cells. To examine the interaction of SMRT with SXR, we carried out mammalian one-hybrid assay in CV-1 cells and immunoprecipitation study in HEK 293 cells.

Key findings: SMRT, but not NCoR suppressed rifampicin-induced SXR-mediated transcription. The SXR-mediated *MDR1* mRNA expression was augmented in the presence of rifampicin, whereas it suppressed the expression following the overexpression of SMRT. In mammalian

one-hybrid assay, only SMRT but not NCoR interacted with SXR on *MDR1* promoter in the presence of rifampicin. In immunoprecipitation study, SMRT bound to SXR regardless of the presence or absence of rifampicin.

Significance: SMRT may be recruited in the SXR-cofactor complex even in the presence of ligand. SMRT may be involved not only in SXR-mediated suppression without ligand, but also in ligand-activated transcription to suppress the overactivation of transcription.

Introduction

Steroid and xenobiotic receptor (SXR), which is also known as pregnane X receptor (PXR), belongs to the nuclear receptor superfamily, and is highly expressed in the liver and small intestine. SXR forms heterodimer with retinoid X receptor (RXR) on xenobiotic-response enhancer modules (XREMs), located in the promoter region of target gene (Bertilsson et al. 1998; Blumberg et al. 1998; Masuyama et al. 2005). The most well-recognized target genes for SXR are cytochrome P450 monooxygenase 3A4 (*CYP3A4*) and P-glycoprotein (P-gp) that is encoded by multidrug resistance (*MDR*) 1 gene (Albermann et al. 2005; Bertilsson et al. 1998; Blumberg et al. 1998; Geick et al. 2001; Gottesman et al. 2002; Loo and Clarke 1999). The *CYP3A4* product is the most abundant CYP and is involved in the metabolism of drugs, steroids, chemicals and environmental procarcinogens (Thummel and Wilkinson 1998). Such broad-specificity of SXR to xenobiotics allows this receptor to play a central role in regulating hepatic drug metabolism. P-gp acts as an ATP-dependent membrane efflux pump to exclude anticancer drugs from tumor cells. It decreases intracellular drug concentrations to confer a multidrug resistance (Gottesman et al. 2002; Hayashi and Tomita 2007; Loo and Clarke 1999). Various nuclear receptor coactivators such as steroid receptor coactivator (SRC)-1 and corepressors such as nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid receptors (SMRT) are known to interact with SXR through ligand binding domain (LBD) to regulate transcription (Chen and Evans 1995; Hörlein et al. 1995; Jones et al. 2000; Takeshita et al. 2001). We have previously reported that SMRT, but not NCoR, suppressed rifampicin-induced transcriptional activity of SXR on *CYP3A4* promoter through specific SMRT-SXR interaction in HepG2 cells (Takeshita et al. 2002). Interestingly, rifampicin also increased the interaction of SXR with SMRT as well as with SRC-1. It is not known, however, whether interaction between liganded SXR and SMRT is specific on *CYP3A* promoter. In the present study, we therefore examined the interaction of SXR with corepressors, NCoR and SMRT, on XREM of *MDR1* promoter.

Materials and methods

Chemicals and plasmids

Rifampicin was purchased from Sigma-Aldrich (St. Louis, MO). Human SXR in pCDG1, mouse NCoR in pCEP4, human SMRT in pCMX, VP16-SXR-LBD (amino acids 107–434), VP16-NCoR-RID (amino acids 1579–2454) and VP16-SMRT-RID (amino acids 1669–2507) were described previously (Takeshita et al. 2002). VP16-SRC-1 (amino acids 595–1440) was described previously (Takeshita et al. 1998). MDR1-thymidine kinase minimum promoter (TK) -LUC, human *MDR1* promoter fragment (nucleotides -7975 to -7013) containing the cluster of xenobiotic-responsive enhancer modules (XREMs) were described previously (Takeshita et al. 2006). FLAG-tagged-SXR in pcDNA3 (Invitrogen, Carlsbad, CA) was described previously (Rokutanda et al. 2008). HA-tagged-SMRT in pcDNA3 was constructed by inserting polymerase chain reaction-generated fragments of human SMRT (amino acids 2098-2507) into *Bam*HI and *Eco*RI sites of pcDNA3, which contains three HA-tags.

Transient cotransfection-based reporter gene assay

LS174T human colon adenocarcinoma-derived clonal cells and CV-1 monkey kidney fibroblast-derived clonal cells were grown in Dulbecco's modified Eagle's medium (DMEM), containing 10 % fetal bovine serum. The serum was stripped of hormones by constant mixing with 5 % (w/v) AG1-X8 resin (Bio-Rad, Hercules, CA) and powdered charcoal before ultrafiltration. Cells were maintained without antibiotics and phenol red. Cells were transiently transfected with expression vectors and reporter vector as shown above using the Lipofectamine 2000 (Invitrogen) in 96 well plates. Cytomegalovirus (CMV)-β-galactosidase plasmid was cotransfected as an internal control. In some samples, empty expression vectors were added to equalize total transfected plasmid concentration. Cells were grown for 24 h with dimethyl sulfoxide (DMSO) as a vehicle or with rifampicin and 0.1% DMSO and then harvested after 16-24 h of incubation. Cell extracts were analyzed for both luciferase, and β-galactosidase activity to correct for transfection efficiency as described previously (Takeshita et al. 2002). All transfection studies were repeated at least three times (n=9; Fig. 1, n=3; Fig. 3). The results shown represent the mean ± standard error of means (S.E.M.).

Semiquantitative RT-PCR

LS174T cells were cultured in 6 well plates and cotransfected with expression vectors encoding SXR and SMRT. The cells were incubated for around 24 h after transfection. Then the culture medium was changed with vehicle (0.1% DMSO) alone or rifampicin with 0.1% DMSO, and harvested after 16-24 h of incubation. Total RNA was extracted from LS174T cells using

TRIzol (Invitrogen) according to manufacturer's instructions. The extracted RNA was treated with DNase I (Invitrogen) to remove any contaminating DNA, and reverse transcribed using PrimeScript RT reagent Kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocol. These samples were analyzed by semiquantitative RT-PCR with C1000 Thermal Cycler (Bio-Rad). The primers used for RT-PCR are as follows: *MDR1*-sense, 5'-CCC ATC ATT GCA ATA GCA GG-3'; *MDR1*-antisense, 5'-GTT CAA ACT TCT GCT CCT GA-3'; *GAPDH*-sense, 5'-ATG GGG AAG GTG AAG GTC GGA GTC A-3'; *GAPDH*-antisense, 5'-CTA CTC CTT GGA GGC CAT GTG GGC C-3'. The thermal cycling was performed with 33 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 40 s. All PCR products were detected by electrophoresis and the intensity of ethidium bromide-stained band was analyzed with Kodak D2100 (Kodak, Norwalk, CT) and Image J software (Ver. 1.41, NIH, Bethesda, MD). The PCR results for each sample were normalized by GAPDH mRNA level as an internal control. All studies were repeated in triplicate. The representative results shown are the mean ± S.E.M.

Preparation of total cell lysate, immunoprecipitation, and Western blotting
Expression vectors containing FLAG-SXR and HA-SMRT were cotransfected into HEK-293 cells using Lipofectamine 2000. Approximately $1 \times 10_5$ cells/cm$_2$ were cultured with or without rifampicin (10 μM). Cells were lysed on ice in CelLytic M (Sigma-Aldrich) containing 1% sodium deoxycholate, cOmplete, Mini; Protease Inhibitor Cocktail Tablet (Roche, Basel, Switzerland), and 1 mM sodium orthovanadate, a phosphatase inhibitor (Wako, Osaka, Japan). Total cell lysate was cleared by centrifugation at 16,400 X*g* for 15 min at 4 °C. For immunoprecipitation, lysate with adjusted protein concentration (Bradford protein assay, Bio-Rad) was mixed with anti-FLAG antibody (Sigma-Aldrich), and incubated end-over-end at 4 °C for 1.5 h. Protein G Sepharose beads (GE Healthcare, Piscataway, NE) were added for 1 h and subsequently washed three times in ice-cold lysis buffer. Proteins were eluted and separated by SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare) and probed with anti-HA antibody (12CA5, Roche). The antibody-antigen complexes were detected using the ECL-Advance system (GE Healthcare) and visualized with a Lumi-Imager imaging analyzer (Roche).

Statistical Analysis
Treatment effects were analyzed using ANOVA. *Post-hoc* comparison was made using Bonferroni's test. The *p*-values < 0.05 were considered to be significant.

Results
Effect of corepressors on SXR-mediated transcription on MDR1 promoter

To examine the effect of SXR and its interaction with corepressors (NCoR and SMRT) on the *MDR1* promoter, we performed transient transfection-based reporter gene assays. MDR1-TK-LUC reporter plasmid and SXR expression plasmid were cotransfected into LS174T cells. It has been reported that *MDR1* expression is induced by rifampicin in LS174T cells (Takeshita et al. 2006). Thus, we used this clonal cell and examined the regulation of *MDR1* gene expression through its XREM by SXR. As shown in Fig. 1, rifampicin activated the transcription on *MDR1* XREM without transfection of SXR, suggesting that rifampicin binds to endogenous SXR. Following transfection of SXR, rifampicin treatment augmented SXR-mediated transcription up to 3-folds. Cotransfection of NCoR did not show any significant changes in the absence or presence of rifampicin. On the other hand, cotransfection of SMRT suppressed not only basal but also rifampicin-activated transcription by SXR. These results suggest that SMRT, but not NCoR, may be involved in SXR-mediated gene suppression both in the absence and presence of rifampicin.

Effect of SMRT on MDR1 mRNA expression
Since rifampicin-induced transcription by SXR was decreased with increased levels of SMRT, we examined further the effect of corepressors on the expression of *MDR1* mRNA in LS174T cells using semi-quantitative RT-PCR (Fig. 2). Without transfection of expression vector for SXR, rifampicin did not alter *MDR1* mRNA expression. Following the transfection of SXR expression vector, *MDR1* mRNA level was significantly augmented by rifampicin, whereas transfection of SMRT expression vector alone showed no effect of rifampicin. Following cotransfection of SXR and SMRT, *MDR1* mRNA level was decreased by rifampicin treatment. Cotransfection of SXR with NCoR did not affect the expression of *MDR1* mRNA in the absence or presence of rifampicin (data not shown). These results indicate that SMRT, but not NCoR, may be involved in SXR-mediated gene suppression even in the presence of rifampicin.

Interaction of SMRT-SXR on XREM in CV-1 cells
Using a mammalian one-hybrid assay in CV-1 cells, we examined the interaction between SXR and receptor interacting domains (RIDs) of cofactors on XREM of *MDR1* gene promoter. Structure of constructs used in this study is shown in Fig. 3A. Without SXR, no transcriptional alteration was observed with any VP16 constructs (Fig. 3B). In the presence of SXR, VP16-SMRT-RID showed significant activation in the absence of rifampicin, suggesting the increase in their interaction. In the presence of rifampicin, transcriptional activity by SXR-VP16-SMRT was augmented further in a dose-dependent manner. Such augmentation was not observed with SXR-VP16-NCoR. These studies indicate that RID of SMRT interacts with SXR on XREM of *MDR1* promoter with or without rifampicin. The increased amount of

rifampicin augmented further the transcription. Indeed, VP16-SRC-1 greatly activated the transcription.

SMRT binds to SXR with and without rifampicin

To examine further the binding of SMRT-SXR, we carried out immunoprecipitation (IP) study using total cell lysate of HEK-293 cells, in which HA-SMRT and FLAG-SXR were over expressed (Fig. 4). IP was performed using anti-FLAG antibody, whereas Western blot was performed using anti-HA antibody. Without HA-SMRT, only non-specific bands were observed (lanes 1 and 2). However, cotransfection of HA-SMRT with FLAG-SXR clearly showed their interaction with or without rifampicin (lanes 3 and 4). Increasing amount of HA-SMRT transfection showed a further augmentation of their interaction (lanes 5 and 6). These results indicate that SMRT binds to SXR regardless of rifampicin in the absence of *MDR1* XREM.

Discussion

In the present study, we showed that SMRT but not NCoR may affect SXR-mediated transcription on XREM of *MDR1* promoter even in the presence of rifampicin in LS174T cells. Cotransfection of SXR and SMRT but not NCoR suppressed the *MDR1* mRNA expression in this clonal cell line. Furthermore, the interaction between SXR and SMRT with or without rifampicin was clarified using both mammalian one-hybrid assay and immunoprecipitation study.

SXR regulates the expression of various genes including *CYP3A4* and *MDR1*. Since we have previously reported that SMRT may be involved in SXR-mediated transcriptional suppression through XREM of *CYP3A4* promoter in HepG2 cells (Takeshita et al. 2002), we analyzed further the effect of SMRT on another SXR-target gene, *MDR1*. Since *MDR1* is highly expressed in intestinal cells (Geick et al. 2001; Gottesman et al. 2002; Loo and Clarke 1999), we used intestine-derived LS174T cells. Transient transfection-based reporter gene assay showed that SMRT suppressed the SXR-mediated transcriptional activation with rifampicin in a dose-dependent manner (Fig. 1). This suppression was not observed when NCoR was expressed. Thus, SMRT may be specifically involved in the suppression of SXR-mediated transcription on XREM of *MDR1* promoter in the presence of rifampicin. These results are similar to those in the previous study using XREM of *CYP3A4* promoter in HepG2 cells (Takeshita et al. 2002). To confirm the role of XREM on *MDR1* gene expression in LS174T cells, we examined the effect of SMRT in SXR-activated *MDR1* mRNA expression in LS174T cells (Fig. 2). Although SMRT transfection alone did not alter *MDR1* mRNA level, transfection of SXR showed a weak but significant increase in its mRNA level. Compared with the result of reporter gene assay, the increase may be much smaller. This could be due to the difference of half-life of *MDR1* mRNA

and luciferase protein. While luciferase protein keeps accumulating until termination of culture, *MDR1* mRNA may be degraded after certain period of time. Following transfection of SMRT together with SXR, rifampicin treatment rather suppressed the expression of *MDR1* mRNA. The cause of such suppression is not fully clarified. However, since mammalian one-hybrid assay and immunoprecipitation study have shown the SMRT-SXR interaction even with rifampicin, such suppression may be caused by relatively greater amount of SMRT compared to those of endogenous coactivators that are responsible for ligand-activated transcription.

As discussed above, our mammalian one-hybrid assay has shown the possible interaction between SXR and SMRT with and without rifampicin but NCoR did not interact with SXR throughout the study (Fig. 3). It has been reported that type II nuclear receptors such as thyroid hormone receptor (TR) bind to NCoR and SMRT in the absence of ligand, and ligand binding results in dissociation of these corepressors (Chen and Evans 1995; Hörlein et al. 1995). In the present study, SXR interacts with SMRT not only without ligand but also with rifampicin. Together with our previous study showing the interaction between SMRT and SXR by rifampicin on *CYP3A4* XREM, SXR can specifically interact with SMRT even in the presence of ligand. Such interaction may inhibit overactivation of SXR-mediated transcription with ligand.

In our immunoprecipitation study, since both SMRT and SXR could not be detected in HEK-293 cells, we transfected HA-SMRT and FLAG-SXR into HEK-293 cells. As shown in Fig. 4, SMRT bound to SXR even in the presence of rifampicin. However, the levels of binding were not altered with or without rifampicin. On the other hand, the binding seems to be reinforced by rifampicin in our mammalian one-hybrid assay. This may be due to the presence of *MDR1* XREM. However, in the mammalian one-hybrid assay, the transcription is activated not only through VP16-coupled SMRT, but also through coactivators that also bind to liganded SXR. Thus, even if the transcription is further augmented by rifampicin in the mammalian one-hybrid assay, we cannot simply interpret such finding as the increase in binding of SMRT to SXR. Nevertheless, we can still conclude that SMRT interacts with liganded SXR on *MDR1* promoter since VP16-SMRT + SXR-mediated transcription is significantly greater than those of VP16 + SXR-mediated transcription.

In our series of studies, although we showed the interaction between SXR and SMRT in the presence of rifampicin on both *CYP3A4* and *MDR1* promoters, other group (Johnson DR et al.) has previously reported that the binding of SXR-SMRT was dissociated by rifampicin in a dose-dependent manner using GST-pull down study and mammalian two-hybrid assay in HEK-293 cells (Johnson et al. 2006). Another group has also shown by mammalian two-hybrid study that paclitaxel dissociated SMRT from SXR (Synold et al. 2001). GST-pull down study is an *in vitro* assay that does not contain nuclear factors, which may affect the SXR-cofactor

interaction. The two-hybrid assay is a potent technique to study the interaction between nuclear receptor and its cofactors. However, the conformation of SXR-LBD may be slightly different from native SXR, because it is fused to GAL4-DBD and thus it does not interact with XREM. On the other hand, they showed that SMRT suppressed the SXR-mediated transcription in HepG2 cells using reporter gene assay. Furthermore, they also showed that suppression of SMRT expression by RNAi caused the upregulation of transcription by rifampicin (Johnson et al. 2006). These results are consistent with our hypothesis that SMRT interacts with SXR even in the presence of rifampicin.

The physiological significance of SMRT interaction with liganded SXR shown in the present and our previous studies has not been fully clarified. SXR is an important component of the body's adaptive defense mechanism against toxic substances including foreign chemicals (xenobiotics) (Kliewer et al. 2002). SXR is activated by a large number of endogenous and exogenous chemicals. Thus, unlike other nuclear receptors, SXR serves as a generalized sensor of hydrophobic toxins. SXR coordinately regulates a large number of genes in the liver and intestine that are involved in all aspects of the detoxification and elimination of xenobiotics from the body (Kliewer et al. 2002). The induction of *CYP3A4* expression represents the basis for an important class of drug-drug interactions in which one drug accelerates the metabolism of other drugs. The induction of *MDR1* expression by one drug accelerates efflux of other drugs. Thus, overactivation of SXR-mediated transcription may produce adverse effect on the metabolism of various endogenous and exogenous substances. Our results together with previous report indicate that the interaction of SMRT with SXR in the presence of ligand may restore such effect.

Conclusion

SMRT interacts with SXR even in the presence of rifampicin. Such interaction may inhibit the SXR-mediated transcription of *MDR1* gene from over-activation in the presence of ligand. With this regard, the SMRT expression may play an important role in regulating ligand-induced transcriptional activity of SXR-target genes.

References

Albermann N, Schmitz-Winnenthal FH, Z'graggen K, Volk C, Hoffmann MM, Haefeli WE, Weiss J. Expression of the drug transporters MDR1/ABCB1, MRP1/ABCC1, MRP2/ABCC2, BCRP/ABCG2, and PXR in peripheral blood mononuclear cells and their relationship with the expression in intestine and liver. Biochem Pharmacol 2005;70:949-58.

Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Bäckman M, Ohlsson R, Postlind H, Blomquist P, Berkenstam A. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. Proc Natl Acad Sci U S A 1998;95:12208-13.

Blumberg B, Sabbagh W Jr, Juguilon H, Bolado J Jr, van Meter CM, Ong ES, Evans RM. SXR, a novel steroid and xenobiotic-sensing nuclear receptor. Genes Dev 1998;12:3195-205.

Chen JD, Evans RM. A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 1995;377:454-7.

Geick A, Eichelbaum M, Burk O. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. J Biol Chem 2001;276:14581-7.

Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2002;2:48-58.

Hayashi M, Tomita M. Mechanistic analysis for drug permeation through intestinal membrane. Drug Metab Pharmacokine 2007;22:67-77.

Hörlein AJ, Näär AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Söderström M, Glass CK, et al. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 1995;377:397-404.

Johnson DR, Li CW, Chen LY, Ghosh JC, Chen JD. Regulation and binding of pregnane X receptor by nuclear receptor corepressor silencing mediator of retinoid and thyroid hormone receptors (SMRT). Mol Pharmacol 2006;69:99-108.

Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, Kliewer SA, Moore JT. The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. Mol Endocrinol 2000;14:27-39.

Kliewer SA, Goodwin B, Willson TM. The Nuclear Pregnane X Receptor: A key regulator of xenobiotic metabolism. Endocr Rev 2002;23:687-702.

Loo TW, Clarke DM. Merck Frosst Award Lecture 1998. Molecular dissection of the human multidrug resistance P-glycoprotein. Biochem Cell Biol 1999;77:11-23.

Masuyama H, Suwaki N, Tateishi Y, Nakatsukasa H, Segawa T, Hiramatsu Y. The pregnane X receptor regulates gene expression in a ligand- and promoter-selective fashion. Mol Endocrinol 2005;19:1170-80.

Rokutanda N, Iwasaki T, Odawara H, Nagaoka R, Miyazaki W, Takeshita A, Koibuchi Y, Horiguchi J, Shimokawa N, Iino Y, Morishita Y, Koibuchi N. Augmentation of estrogen

receptor-mediated transcription by steroid and xenobiotic receptor. Endocrine 2008;33:305-16.

Synold TW, Dussault I, Forman BM. The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. Nat Med 2001;7:584-90.

Takeshita A, Yen PM, Ikeda M, Cardona GR, Liu Y, Koibuchi N, Norwitz ER, Chin WW. Thyroid hormone response elements differentially modulate the interactions of thyroid hormone receptors with two receptor binding domains in the steroid receptor coactivator-1. J Biol Chem1998;273:21554-62.

Takeshita A, Koibuchi N, Oka J, Taguchi M, Shishiba Y, Ozawa Y. Bisphenol-A, an environmental estrogen, activates the human orphan nuclear receptor, steroid and xenobiotic receptor-mediated transcription. Eur J Endocrinol 2001;145:513-7.

Takeshita A, Taguchi M, Koibuchi N, Ozawa Y. Putative role of the orphan nuclear receptor SXR (steroid and xenobiotic receptor) in the mechanism of CYP3A4 inhibition by xenobiotics. J Biol Chem 2002;277:32453-8.

Takeshita A, Inagaki K, Igarashi-Migitaka J, Ozawa Y, Koibuchi N. The endocrine disrupting chemical, diethylhexyl phthalate, activates MDR1 gene expression in human colon cancer LS174T cells. J Endocrinol 2006;190:897-902.

Thummel KE, Wilkinson GR. In vitro and in vivo drug interactions involving human CYP3A. Annu Rev Pharmacol Toxicol 1998;38:389-430.

Figure Legends

Fig. 1. Suppression of SXR-mediated transcription through XREM of *MDR1* promoter by SMRT.

Expression plasmid encoding SXR (0.01 μg) was cotransfected with the reporter plasmid MDR1-TK-LUC (0.15 μg) and CMV-β-galactosidase control vector (0.005 μg), and increasing amounts (0.002, 0.01, 0.05 μg) of expression plasmid encoding NCoR or SMRT into LS174T cells. Cells were incubated with or without 10 μM rifampicin for 24 h and analyzed for luciferase activity. Luciferase activity was normalized to β –galactosidase activity and then calculated as relative luciferase activity. Data represent means ± S.E.M. * $P < 0.05$ by ANOVA compared with SXR (+), NCoR (-), SMRT (-), and rifampicin (+).

Fig. 2. Suppression of SXR-mediated expression of intrinsic *MDR1* mRNA in LS174T cells by SMRT.

Expression plasmid encoding SXR (0.2 μg) was cotransfected with the expression plasmid encoding SMRT (2.2 μg) into LS174T cells. Cells were incubated with or without 10 μM rifampicin for 12-16 h. cDNAs were obtained from total RNAs from each plate with reverse transcriptase. Semiquantitative RT-PCR was performed with primers indicated in Material and methods. The expression levels of *MDR1* mRNA were normalized with *GAPDH* mRNA. Data

shown are relative *MDR1* mRNA levels as SXR (-), SMRT (-), and rifampicin (-) as 1. The data represent means ± S.E.M. * $P < 0.05$ by ANOVA.

Fig. 3. Interaction between SXR and cofactors in the presence of XREM in CV-1 cells.

A. Schematic representative of the method of mammalian one-hybrid assay and constructs that were used in the present study. B. Expression plasmids encoding SXR (0.01 μg) and either VP16 alone, VP16-NCoR-RID, VP16-SMRT-RID, or VP16-SRC-1-RID (0.05 μg) were cotransfected with MDR1-TK-LUC reporter plasmid (0.15 μg) (and the CMV-β-galactosidase control vector (0.01 μg)) into CV-1 cells. Cells were then treated with 1 μM or 10 μM rifampicin or without ligand for 24 h and analyzed for luciferase activity. Luciferase activity was normalized to β-galactosidase activity and then calculated as -fold luciferase activity, with 1-fold basal activity defined as the luciferase activity with VP16 empty vector in the absence of ligand. Data represent means ± S.E.M. ** $P < 0.01$ by ANOVA, compared with SXR (+), VP16 alone, rifampicin (10 μM) vs. SXR (+), VP16-SMRT, rifampicin (10 μM); * $P < 0.05$ compared with SXR (+), VP16 alone, rifampicin (-) vs. SXR (+), VP16-SMRT, and rifampicin (-).

Fig. 4. SMRT binds to SXR regardless of rifampicin.

Increasing the amount (0, 0.5, 1 μg) of expression vectors encoding HA-SMRT and FLAG-SXR (1 μg) were cotransfected into HEK-293 cells. Total amounts of DNA for each well were balanced by adding an empty vector. Cells were cultured in the absence or presence of rifampicin (10 μM) for 40 min. Following cell lysis, equal amounts of cell lysates were subjected to immunoprecipitation with anti-FLAG antibody and immunoprecipitates were analyzed by Western blotting with anti-HA antibody. Representative blots from three replicates are shown. The position of standard molecular masses (kDa) is indicated.

Fold Basal Luciferase Activity

- Rif
+ Rif 1 μM
+ Rif 10 μM

VP16
VP16-NCoR
VP16-SMRT
VP16-SRC-1
SXR + VP16
SXR + VP16-NCoR
SXR + VP16-SMRT
SXR + VP16-SRC-1

*
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IP Anti-FLAG / WB Anti-HA

HA-SMRT → 50 kDa

FLAG-SXR	+	+	+	+	+	+
HA-SMRT	-	-	0.5	0.5	1	1
rifampicin	-	+	-	+	-	+