1,25-dihydroxyvitamin D3 and its receptor inhibit the chenodeoxycholic acid-dependent transactivation by farnesoid X receptor

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Abstract

Farnesoid X receptor (FXR), the receptor for bile acids, including chenodeoxycholic acid (CDCA), is a member of the nuclear receptor superfamily, which also includes the receptors for retinoic acid, vitamin D (D3), thyroid hormone, thiazolidinedione and 22(*R*)-hydroxycholesterol. Here, we have evaluated the effects of a series of ligands and their receptors on the promoter activity induced by CDCA/FXR. The kidney cell line, CV1, was cotransfected with FXR-expression plasmid and the luciferase-based reporter gene that has a thymidine kinase promoter fused to the canonical FXR-responsive element or the natural promoter for the small heterodimer partner (SHP), bile salt export pump (BSEP), and ileum bile acid (I-BABP) gene. D3 and its receptor (VDR) inhibited the transactivation of all four reporter constructs that are enhanced by CDCA/FXR. The effect of D3 on the expression of the BSEP and SHP genes in HepG2 cells and that of the I-BABP gene in Caco-2 cells were confirmed by reverse transcription (RT)–PCR. Deletion analysis of VDR revealed that its ligand-binding domain (LBD) is responsible for the repression and the DNA-binding domain (DBD) is dispensable. Specific interaction between FXR and VDR was detected with the *in vitro* pull-down assay using chimeric FXR or VDR fused to glutathione-S-transferase.

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Introduction

Farnesoid X receptor (FXR) functions as the receptor for bile acids such as chenodeoxycholic acid (CDCA) (Chawla *et al.* 2001). In the liver, bile acid is synthesized from cholesterol with cholesterol 7α-hydroxylase (CYP7A1) as the rate-limiting enzyme (Chawla *et al.* 2001). The expression of CYP7A1 is negatively regulated by the transcription factor small heterodimer partner (SHP), the expression of which is enhanced by the ligand-bound FXR. Consequently, bile acid suppresses the expression of CYP7A1 *in vivo* via FXR, resulting in a decrease in its own production. On the other hand, the metabolism of bile acid is precisely regulated in the enterohepatic circulation, where the bile salt export pump (BSEP) and the ileum bile acid transporter (I-BABP) play critical roles. BSEP mediates the ATP-dependent transport of bile salts across the canalicular membrane of hepatocytes (Ananthanarayanan *et al.* 2001, Plass *et al.* 2002), whereas I-BABP, which is a cytosolic protein expressed in the ileum, binds bile acid with a high affinity to mediate its cytosolic transportation (Grober *et al.* 1999). Short DNA sequences, referred to as FXR-responsive elements (FXREs), are present in the promoters of the SHP (Goodwin *et al.* 2000, Lu *et al.* 2000), BSEP (Ananthanarayanan *et al.* 2001) and I-BABP genes (Grober *et al.* 1999). On the FXREs, FXR forms a heterodimer with retinoid X receptor (RXR) and stimulates transcription in a bile acid-dependent manner. 22(*R*)-hydroxycholesterol (22(*R*)-HC) (Janowski *et al.* 1996) is the ligand for liver X receptor α (LXRα) (Willy *et al.* 1995). 22(*R*)-HC and LXRα directly stimulate the LXR-responsive element in CYP7A1 gene, while liganded FXR downregulates the transcription of CYP7A1 by the induction of SHP (Lu *et al.* 2000).

Small lipophilic hormones and ligands, such as retinoic acid (RA), 1,25-dihydroxyvitamin D_3 (D3), thyroid hormones (tri-iodothyronine (T_3) and thyroxine (T_4)), thiazolidinedione (TZD), and oxysterol, bind and activate RA receptor (RAR), D3 receptor (VDR), T_3 receptor (TR), peroxisome proliferator-activated receptor γ2 (PPARγ2) and LXRα respectively (McKenna *et al.* 2002). It should be noted that the synthesis and enterohepatic circulation of bile acid have profound effects on the absorption and metabolism of these lipophilic ligands from the intestine. For example, severe malabsorption of D3 and vitamin A (a precursor of RA) has been reported in patients with progressive familial intrahepatic cholestasis

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type 2 (PFIC2) (Whitington *et al.* 1994), in which a genetic abnormality of the BSEP causes the impairment of bile acid excretion from hepatocytes (Thompson & Strautnieks 2001). The enterohepatic circulation of bile acid also affects the intestinal uptake of T_4 (DiStefano *et al.* 1993) and TZD (Kawai *et al.* 2000). In this study, we evaluated the effects of the liganded receptors on the CDCA/FXR-dependent transactivation of the reporter genes, including canonical FXRE fused to the thymidine kinase (tk) promoter and the natural promoters for BSEP, I-BABP or SHP. We observed that VDR suppresses the transactivation driven by CDCA/FXR in a D3-dependent manner. Glutathione-S-transferase (GST) pull-down assay revealed the specific interaction between FXR and VDR.

Materials and Methods

Plasmids

With human genomic DNA as the template, the BSEP promoter regions of the BSEP gene (–165 to +8) (Ananthanarayanan *et al.* 2001) and the SHP gene (–332 to +10) (Lee *et al.* 1998) were amplified by PCR. Both PCR products were digested with NheI and HindIII, and subcloned into pGL2-Basic Vector (Promega) to generate hBSEP-Luc and hSHP-Luc. The parental plasmid (pCMX) for rat FXR (pCMX-rFXR), human RARα (pCMX-hRARα), human TRβ1 (pCMX-hTRβ1) (Umesono *et al.* 1991) and human LXRα (pCMX-hLXRα) (Willy *et al.* 1995) is driven by the cytomegalovirus (CMV) promoter (Umesono *et al.* 1991). The parental plasmid (pSG5) for human VDR (pSG5-hVDR) and mouse PPARγ2 (pSG5-mPPARγ2) is driven by the SV40 early promoter fused with the rabbit β-globin intron III (Green *et al.* 1988). The validity of these expression plasmids was confirmed by the reporter assay using the reporter plasmids that have the canonical hormone-responsive elements (Sasaki *et al.* 1995, Kawai *et al.* 2004 and data not shown). To prepare VDR-deletion constructs, the cDNA for human VDR (pSG5-hVDR) was amplified by PCR with the specific primers to generate PCR products including VDR-D1 (codon 20–427), D2 (codon 89–427) and D3 (codon 20–125). These DNA fragments were digested with EcoRI and BamHI and ligated into the mammalian expression plasmid, pSG5, to generate pSG5-hVDR-D1, D2 and D3. DNA sequencing was performed to confirm the identity of all the constructed plasmids. Dr Makoto Makishima (Nippon University, Japan) provided GST-FXR, in which the ligand-binding domain (LBD) of human FXR (codon 193–472) was fused to the open reading frame of GST cDNA in pGEX-4T-1 plasmid (Pharmacia). GST-VDR that had full-length VDR (1–427) was a gift from Dr Rajiv Kumar (Mayo Clinic and Foundation, USA) (Craig & Kumar 1996).

Cell culture and transient transfection

CV1 cells and HepG2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin G (100 units/ml) and streptomycin (100 μg/ml) at 37 °C under 5%/95% air. By the calcium-phosphate technique (Sasaki *et al.* 1995), the cells were cotransfected with 1·8 μg reporter gene, 3·6 μg β-galactosidase expression vector (Sasaki *et al.* 1995), and the expression plasmids for the receptors. HepG2 cells were transfected with 700 ng pCMX-BSEP-Luc, 70 ng expression plasmid (pCMX-rFXR) (Forman *et al.* 1995), 1 μg β-galactosidase expression vector and 300 ng empty vector per well by the lipofection method (Gibco-BRL). After transfection for 24 h (CV1) or 5 h (HepG2), the medium was replaced with fresh DMEM containing 10% dextran-charcoal-stripped FBS (10% DCC serum) in the presence or absence of 1 μM RA, T_3, D3 or 5 μM troglitazone, or 22(*R*)-HC. After 24-h incubation, the cells were harvested, and the luciferase activity was measured. Transfection efficiencies were normalized by a β-galactosidase assay.

GST pull-down assay

E. coli (DH5α) transformed with the GST-FXR or GST-VDR were induced with 0·1 mM isopropyl-1-thio-β-galactopyranoside (ITGP) for 4 h. The *E. coli* pellet was sonicated and the fusion proteins were mixed with glutathione-Sepharose beads (Amersham Pharmacia Biotech) for purification. Receptor proteins including RARα, TRβ1, PPARγ2, LXRα and VDR were *in vitro* translated with rabbit reticulocyte lysates (Promega) in the presence of ^{35}S-methionine. Radiolabeled receptors were incubated with GST fusion proteins (approximately 1 μg/sample) in the binding buffers for GST-FXR (140 mM KCl, 20 mM Tris HCl (pH 7·5), 0·05% Nonidet P-40 (NP-40), 0·5 mM phenilmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol) or for GST-VDR (150 mM NaCl, 20 mM Tris HCl (pH 7·5), 0·3% NP-40, 1 mM DTT, 0·5 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml aprotinin) for 3 h at 4 °C, and washed with the binding buffer three times. Bound protein was analyzed by 10–14% SDS–PAGE and visualized by the BAS-1000 autoradiography system (Fuji Film, Tokyo, Japan) (Kawai *et al.* 2004).

RNA isolation, cDNA synthesis and quantitative real-time detection PCR (RT–PCR)

RNA isolation from HepG2 cells and cDNA synthesis were performed as described previously (Kawai *et al.* 2004). The cDNA for BSEP was amplified with the forward primer 5′-CCACTTCTGCCTTAGACACA-3′ and the reverse primer 5′-CATGACAGCAATGATAT CCG-3′ (134 bp PCR product). The cDNA for the human SHP gene was amplified with the forward primer

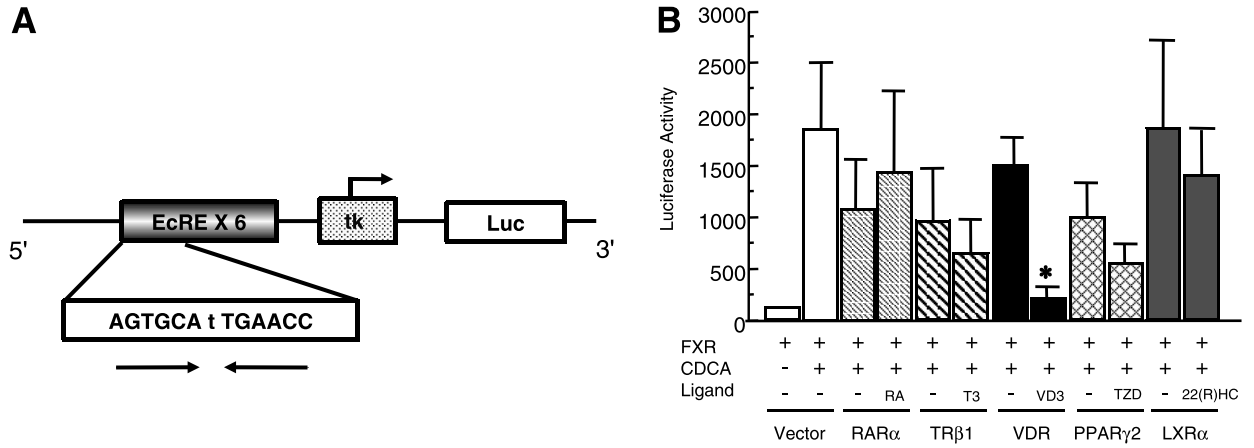

Figure 1 CDCA-dependent transactivation of EcRE by FXR is inhibited by D3-bound VDR. (A) Schematic representation of $(EcRE)_6$-tk-Luc reporter plasmid. (B) CV1 cells were cotransfected with 1·8 μg $(EcRE)_6$-tk-Luc reporter plasmid and 180 ng expression plasmid for rat FXR (pCMX-rFXR) along with 180 ng empty vector (open bar) or the expression plasmids for RARα (hatched bar), TRβ1 (filled, hatched bar), VDR (solid bar), PPARγ2 (cross-hatched bar) and LXRα (dark grey bar). Cells were treated with or without 100 μM CDCA in the presence or absence of 1 μM RA, T_3, D_3 or 5 μM troglitazone or 22(*R*)-HC as indicated. The data represent the mean ± S.D. of five individual transfection experiments. **P*<0·05 versus without ligands.

5′-ggaatatgcctgcctgaaag-3′ and the reverse primer 5′-tcggaatggacttgagggt-3′ (194 bp PCR product). The cDNA for the human I-BABP gene was amplified with the forward primer 5′-tcacttggtcccagcacta-3′ and the reverse primer 5′-cttgtcacccacgatctct-3′ (182 bp PCR product). The β-actin cDNA was amplified with the forward primer (5′-GGGCATGGGTCAGAAGGATT-3′) and the reverse primer (5′-GAGGCGTACAGGGA TAGCAC-3′) to generate a 302 bp product. We designed the primer sets to encompass introns 27, 1 and 3 in the genome of the BSEP, SHP and I-BABP gene respectively. Real-time detection PCR was performed with Light-Cycler (Roche), and the optimal buffer conditions were determined for each template. The parameters for the PCR amplification of the BSEP, SHP and I-BABP genes were denaturation at 95 °C for 10 s and annealing at 62 °C for 10 s, followed by elongation at 72 °C for 5 s.

Statistical analysis

Each experiment was performed in duplicate and repeated more than three different times; each result is expressed as the mean ± S.D. Statistical significance was determined with the analysis of variance (ANOVA) and Fisher's protected least significant difference (PLSD) test. $P<0·05$ was considered to be significant.

Results

The effect of nuclear receptors and their ligands on the transactivation by FXR/CDCA

We initially utilized the luciferase reporter gene in which the thymidine kinase (tk) promoter is fused to six copies of FXRE, originally identified as the ecdysone response element (EcRE) in the insect hsp27 gene (Fig. 1A) (Forman *et al.* 1995). This artificial reporter gene $(EcRE)_6$-tk-Luc, and the FXR expression plasmid (pCMX-rFXR) (Forman *et al.* 1995) were cotransfected into CV1 cells together with the expression plasmids for RARα, TRβ1, VDR, PPARγ2 or LXRα. It should be noted that, without FXR or CDCA, $(EcRE)_6$-tk-Luc was not transactivated by liganded or unliganded RARα, TRβ1, VDR, PPARγ2 or LXRα (data not shown). As reported, CDCA and FXR stimulated the luciferase activity (Fig. 1B). In the presence of D3, VDR exhibited a potent suppressive effect on the luciferase activity of $(EcRE)_6$-tk-Luc, whereas the effects of other ligands/receptors were not statistically significant. Subsequently, we prepared reporter genes in which the luciferase gene was fused to the natural promoter of BSEP, SHP and I-BABP (Fig. 2A). In the presence of CDCA, FXR enhanced the transcriptional activities of these promoters in CV1 cells. Interestingly, ligand-dependent transcriptional repression by VDR was again detected in all three promoters (Fig. 2B–D). Although bile acid is known to bind not only with FXR but also with VDR (Makishima *et al.* 2002), VDR without D3 did not affect the basal transactivation by FXR/CDCA, presumably due to the low affinity of CDCA for VDR (Makishima *et al.* 2002). TZD-bound PPARγ2 inhibited the I-BABP promoter (Fig. 2D), whereas its inhibition of the promoter of BSEP or SHP was not significant (Fig. 2B and C). Unliganded TR suppressed the I-BABP promoter stimulated by CDCA/FXR, and the inhibition was released by T_3 (Fig. 2D). Although this suggested the presence of a cryptic T_3-responsive element (TRE) in the I-BABP promoter, I-BABP-Luc was not stimulated by liganded-TRβ1 in the absence of FXR/CDCA (data not shown).

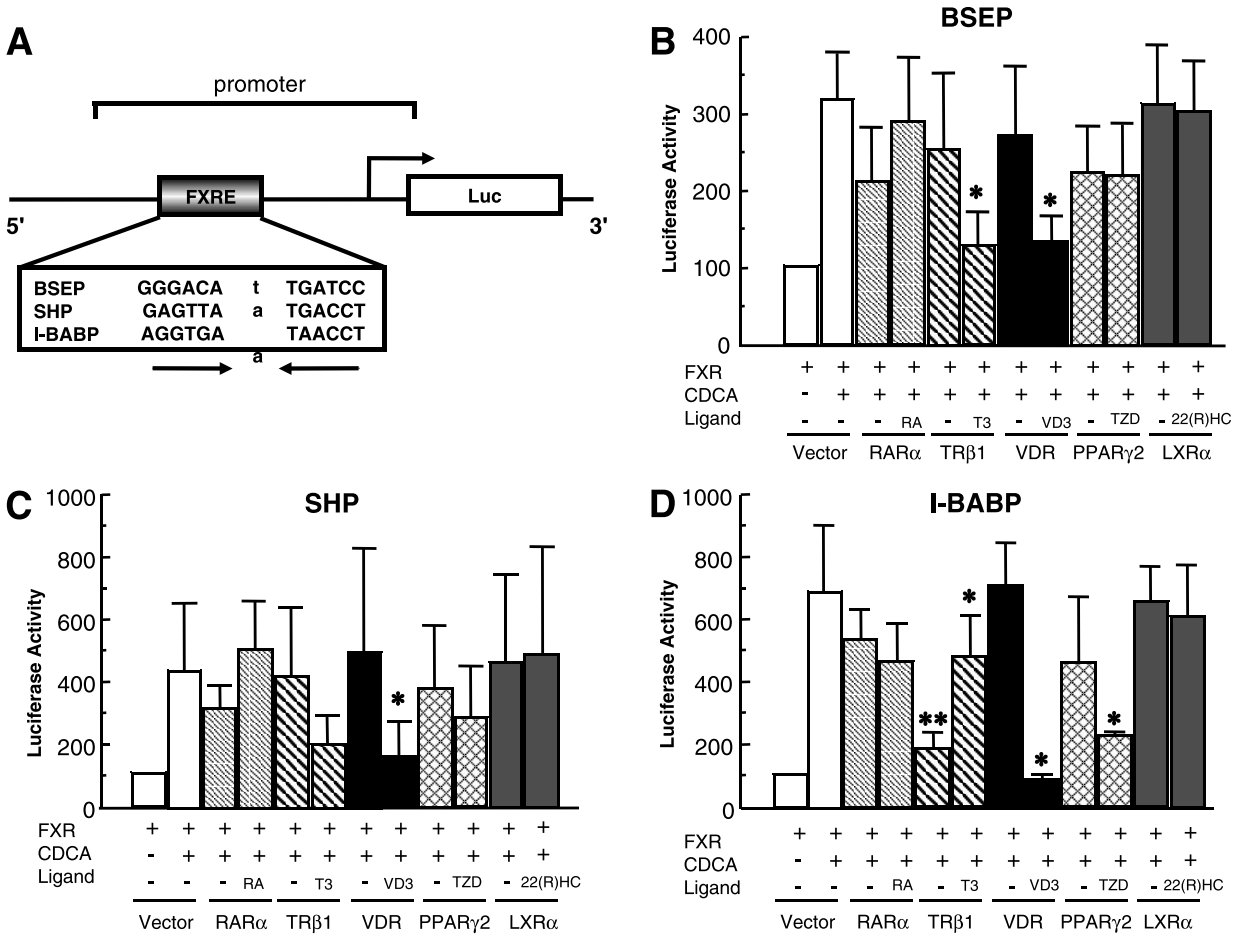

Figure 2 The promoters for BSEP, SHP and I-BABP are transactivated by CDCA/FXR and inhibited by D3-bound VDR. (A) Schematic representation of FXREs in the reporter plasmids. (B–D) CV1 cells were cotransfected with 1·8 μg reporter plasmid for BSEP (B), SHP (C) or I-BABP (D), and 180 ng pCMX-rFXR along with 180 ng empty vector (open bar) or the expression plasmids for RARα (hatched bar), TRβ1 (filled, hatched bar), VDR (solid bar), PPARγ2 (cross-hatched bar) and LXRα (dark grey bar). The data represent the mean ± S.D. of five individual transfection experiments. **P*<0·05 versus without ligands. ***P*<0·05 CDCA/FXR versus CDCA/FXR and TR.

In other combinations, the difference of transcriptional activity in the presence or absence of ligands was not significant. It was reported that the SHP and I-BABP genes are regulated by LXRα (Goodwin *et al*. 2003) and PPARγ2 (Landrier *et al*. 2005) respectively. As shown in Fig. 2C and D, however, liganded LXRα and PPARγ2 did not affect the activities of these promoters driven by FXR/CDCA. Without FXR or CDCA, BSEP-, SHP- or IBABP-Luc was not affected by the RARα, TRβ1, VDR, PPARγ2 or LXRα in the presence or absence of cognate ligands (data not shown).

VDR-DBD is not required for the repression of the transactivation of BSEP promoter by CDCA/FXR

Since the expression of BSEP is known to be strictly regulated by FXR (Wagner *et al*. 2003), we focused on the inhibition of the BSEP promoter by D3/VDR in the liver. In the absence of VDR expression, D3 did not exhibit a repressive effect (data not shown), excluding the possibility that D3 may affect the function of FXR. The transactivation of the BSEP promoter by CDCA/FXR was suppressed in a D3-dependent manner (Fig. 3A). The half-maximal inhibition was approximately 1–10 nM. To map the VDR region involved in this cross-talk, we generated three deletion mutants (Fig. 3B): VDR-D1 lacking the A/B region, VDR-D2 lacking both the A/B region and DNA-binding domain (DBD), and VDR-D3 lacking the A/B region and the LBD. All the constructs were designed to harbor the hinge region (D-domain) that is required for nuclear localization (Luo *et al*. 1994). Interestingly, D3-dependent transcriptional repression was observed not only in VDR-D1 but also in VDR-D2 that lacks DBD (Fig. 3C). These results indicate that VDR-LBD is sufficient for the repression of the transactivation of BSEP promoter by CDCA/FXR.

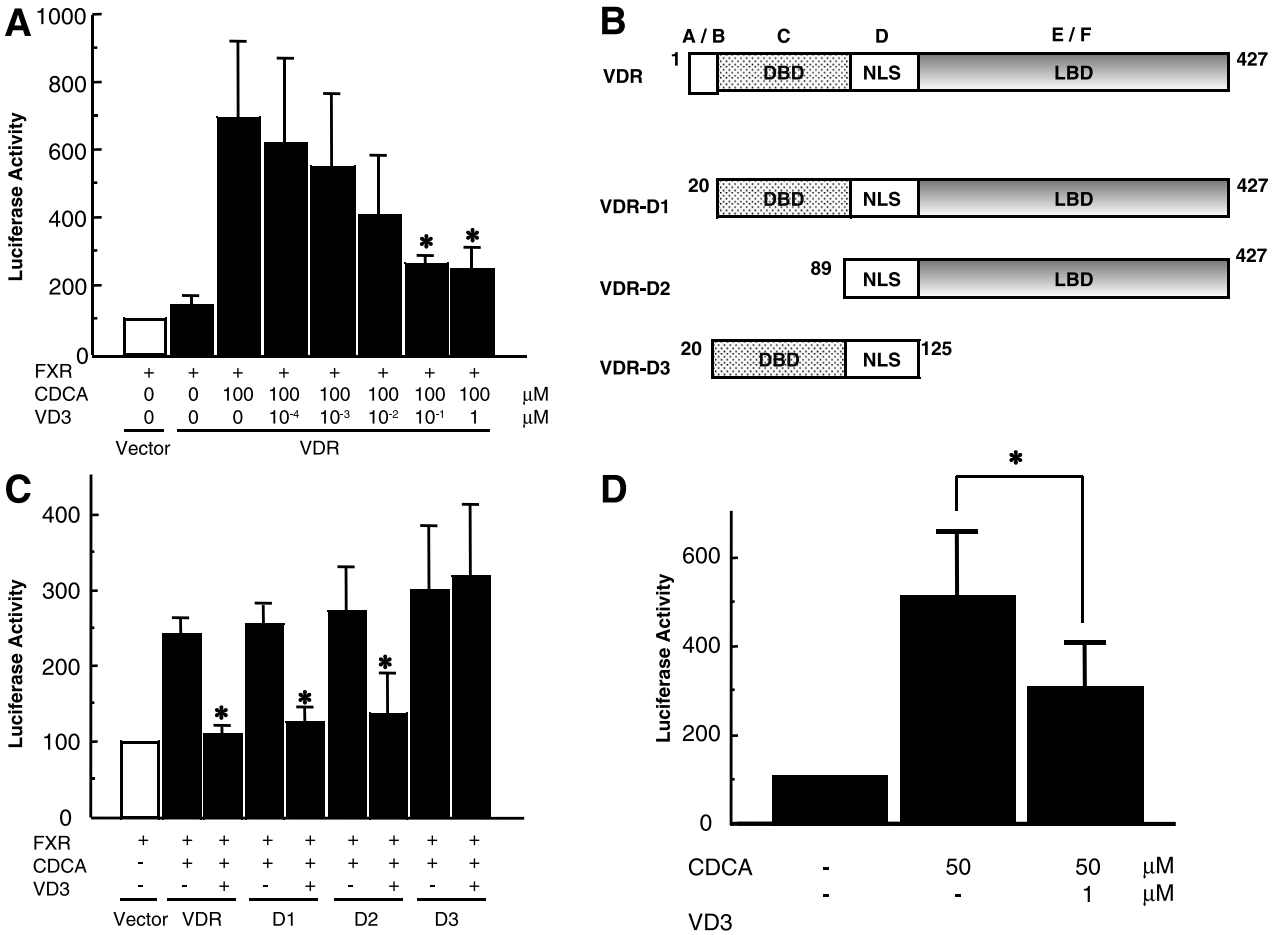

Figure 3 (A) CDCA/FXR-dependent transactivation of the BSEP promoter is repressed by VDR at the physiologic concentration of D3 and mediated through its LBD, but not DBD. The transcriptional activity of the BSEP promoter is stimulated by CDCA/FXR and suppressed by VDR in a D3-dependent manner. CV1 cells were cotransfected with 1·8 μg BSEP-Luc reporter plasmid and the expression plasmids for FXR and VDR (180 ng each). (B) Schematic representation of VDR deletion mutants. Numbers above the schema represent the position in amino-acid sequence. NLS: nuclear localization signal. (C) VDR-DBD is not indispensable for the D3-dependent inhibition of the BSEP promoter driven by CDCA/FXR. The data represent the mean ± S.D. of three individual transfection experiments. *P<0·05 versus without D3. (D) Endogenous VDR in HepG2 cells inhibits the transactivation of BSEP promoter stimulated by CDCA and FXR. HepG2 cells were cotransfected with 700 ng BSEP-Luc reporter plasmid and 70 ng expression plasmid for FXR. Cells were treated with or without 50 μM CDCA in the presence or absence of 1 μM D3. The data represent the mean ± S.D. of five individual transfection experiments. *P<0·05 versus without D3.

Because the hepatic expression of VDR is not so high, we wanted to determine whether endogenous VDR is able to mediate the D3-dependent inhibition. To this end, we cotransfected BSEP-Luc and the expression plasmid for FXR into human hepatoblastoma cells, HepG2, and observed that the CDCA-dependent transactivation is repressed by the administration of D3 (Fig. 3D). This suggests that the suppressive effect by D3 is mediated via the endogenous VDR in HepG2 cells.

VDR specifically interacts with FXR in vitro

To explore the molecular mechanism of D3/VDR-dependent inhibition, we generated GST-FXR and GST-VDR in which GST protein was fused to FXR-LBD or full-length VDR (Fig. 4A), and performed GST pull-down assay (Fig. 4B–D). In the presence of CDCA and specific ligands for receptors studied in Figs 1 and 2, VDR, but not RARα, PPARγ2 or LXRα, interacted with GST-FXR. A weak interaction between TRβ1 and GST-FXR was also detected. As Fig. 4C shows, the interaction between VDR and FXR is independent of D3. We also performed a reciprocal experiment with ^{35}S-labeled FXR and the GST-VDR, in which GST was fused with full-length human VDR. Fig. 4D shows that the radiolabeled FXR interacted with the GST-VDR in a D3-independent fashion.

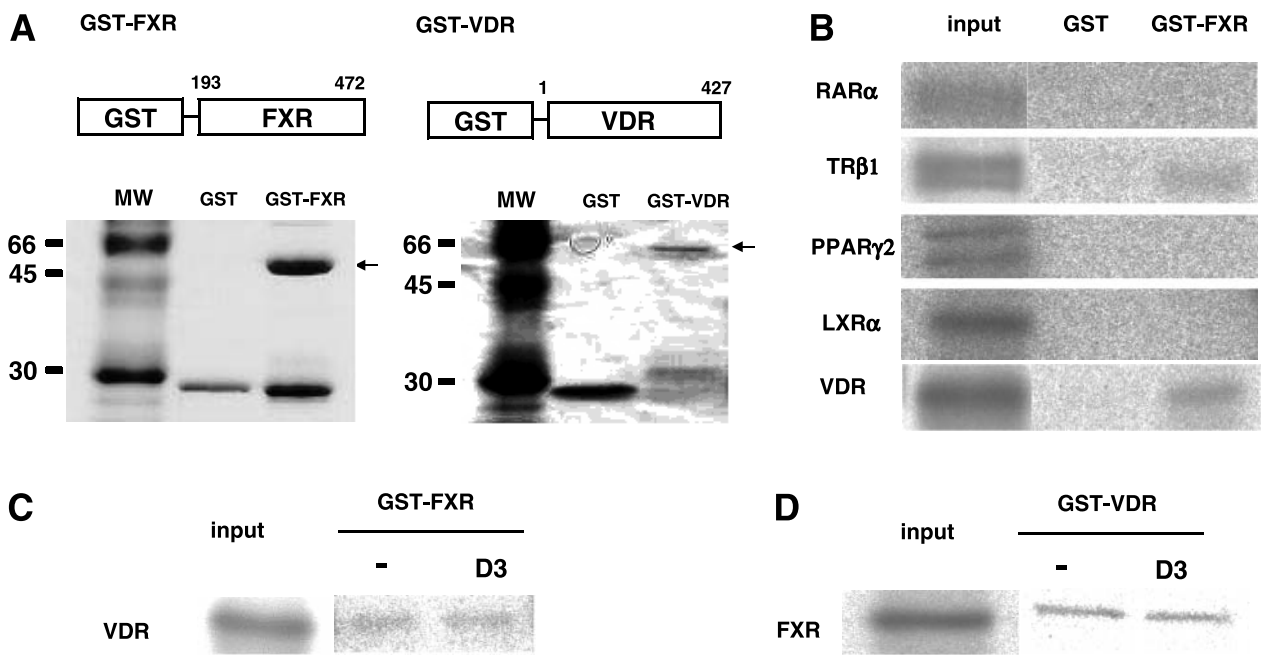

Figure 4 FXR interacts with VDR in a D3-independent manner. (A) Schematic representation of GST-FXR and VDR (upper panels) and Coomassie blue staining for these fusion proteins expressed in *E. coli* (lower panel). Arrows indicate the position of GST-FXR and VDR. (B) FXR-LBD interacts with VDR and partially with TRβ1, but not with RARα, PPARγ2 and LXRα. GST-FXR was incubated with ^{35}S-labeled receptors in the presence of 100 μM CDCA and cognate ligands, that is, RA (1 μM), T_3 (1 μM), D3 (1 μM), troglitazone (5 μM) and 22(*R*)-HC (5 μM). (C and D) The interaction between FXR and VDR is D3-independent. Input and bound fractions are indicated as 'input' and 'GST-FXR or VDR' respectively. In the absence or presence of 1 μM D3, GST-FXR or GST-VDR was incubated with ^{35}S-labeled VDR (C) or FXR respectively (D). In all binding reactions, 100 μM CDCA (final concentration) was added.

Endogenous expression of the BSEP and SHP genes in HepG2 cells and the I-BABP gene in Caco-2 cells stimulated by CDCA is suppressed by D3

We investigated the effect of D3/VDR on BSEP mRNA, using real-time RT–PCR. As shown in Fig. 5A and B, the BSEP mRNA was stimulated by the administration of CDCA and was reduced to approximately 40% in the presence of D3. A similar result was obtained from RT–PCR for the SHP gene in HepG2 cells (Fig. 5C (lanes 1–3) and D). Caco-2 cells are known to have the endogenous expression of the I-BABP, VDR and FXR genes. We studied the expression of the I-BABP gene in this cell line, and found that addition of 1 μM D3 inhibited the I-BABP gene activated by CDCA (Fig. 5C (lanes 4–6) and E). These results suggest that D3 suppresses the CDCA/FXR-dependent expressions of the BSEP and SHP genes in HepG2 cells and that of the I-BABP gene in Caco2 cells.

Discussion

We have shown that D3-bound VDR inhibits the transactivation driven by CDCA/FXR. Since D3/VDR suppressed the CDCA/FXR-dependent transactivation of canonical FXRE fused to the heterologous tk promoter (Fig. 1B), liganded VDR was believed to target directly the transactivation function of FXR. However, the molecular mechanism of the ligand-dependent inhibition of transcription by nuclear receptors, including D3/VDR, has been poorly understood. The squelching of RXR, p160 family and CBP/p300 does not account for this inhibition, because not only VDR but also TRβ1, RARα, LXRα and PPARγ2 associate with these nuclear factors. In agreement with this, D3/VDR-dependent repression was not affected by the overexpression of SRC1, CBP, p300 and the deletion mutant of p300 that has the N-terminal receptor-interacting domain but lacks the HAT domain (Ogryzko *et al.* 1996) (data not shown). Recently, FXR has been reported to associate with non-HAT coactivators, including TRAP220 (Pineda Torra *et al.* 2004) and PGC1 (Savkur *et al.* 2005*a*), both of which also interact with VDR as well as TRβ1 (McKenna & O'Malley 2002, Zhang *et al.* 2004, Savkur *et al.* 2005*b*). However, the overexpression of wild-type PGC1 or wild- or dominant negative-type TRAP220 (Yuan *et al.* 1998) had no effect on D3/VDR-dependent inhibition (data not shown). On the other hand, VDR has been recently reported to associate with the Williams syndrome transcription factor (WSTF) that has ATP-dependent chromatin-remodeling activity (Kitagawa *et al.* 2003). Moreover, Yanagisawa *et al.* (2002) reported that GCN5

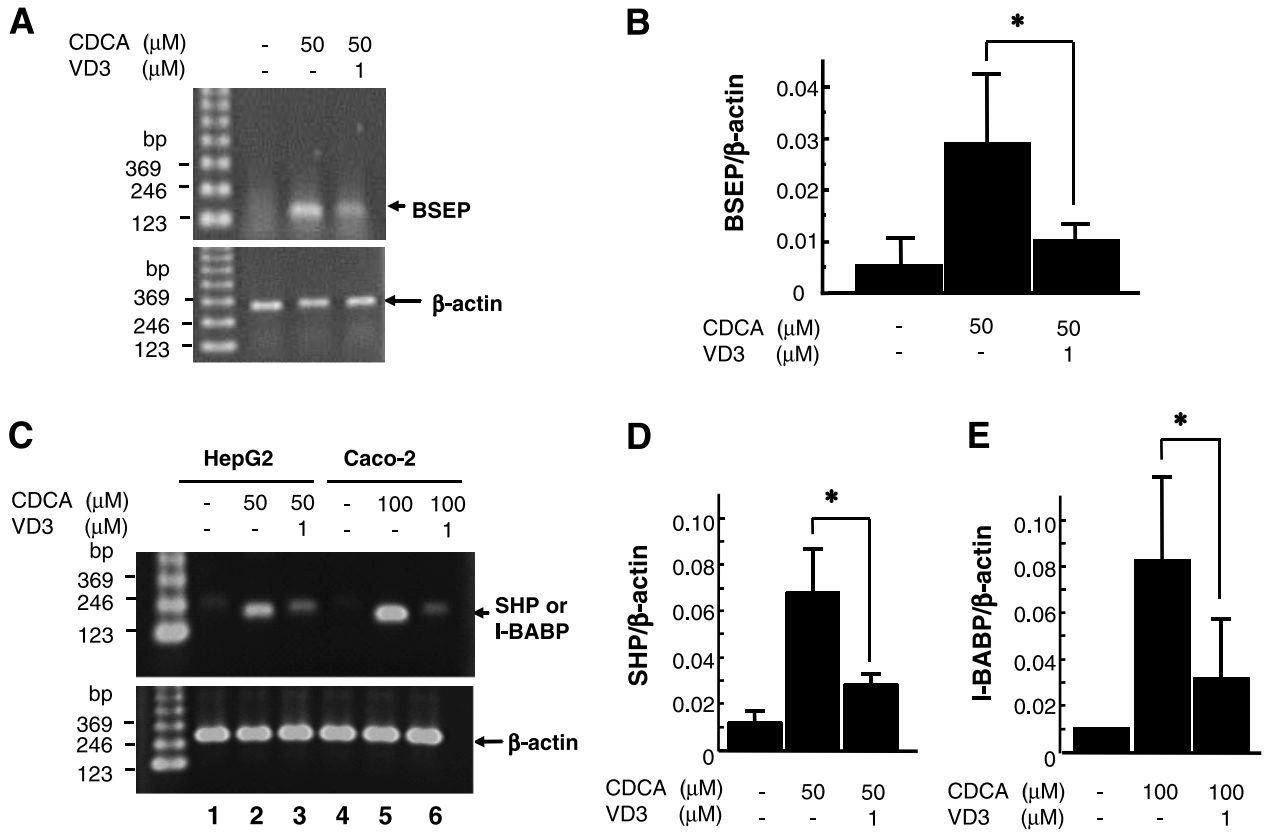

Figure 5 Endogenous expressions of the BSEP and SHP genes in HepG2 cells and the I-BABP gene in Caco-2 cells transactivated by CDCA are suppressed by D3. (A) RT–PCR was performed with BSEP and β-actin mRNA isolated from HepG2 cells treated with or without 50 μM CDCA and/or 1 μM D3 for 24 h. For gel electrophoresis, 33 cycles were employed for the amplification of BSEP and 17 cycles for β-actin. The PCR products were subjected to a 1·4% agarose gel and stained with ethidium bromide for the photographs. (B) BSEP mRNA expression was evaluated by the quantitative real-time detection RT–PCR. (C–E) RT–PCR of the SHP and I-BABP mRNAs. In the presence of indicated ligand concentration, the mRNA for SHP or I-BABP was isolated from HepG2 cells or Caco-2 cells respectively. The mRNAs were analyzed by gel electrophoresis (C) and by real-time PCR (D and E). The amount of PCR product was normalized with that of β-actin. The experiments were repeated three times, and the data were expressed as the mean ± S.D. *$P<0{\cdot}05$.

and TRRAP/PAF400 enhance the ligand-dependent transactivation by multiple receptors including VDR. The association of these cofactors with FXR remains to be investigated. The D3/VDR-mediated negative regulation has been reported in several genes, including parathyroid hormone (PTH) (Demay *et al.* 1992), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Towers *et al.* 1998) and 25-hydroxy-vitamin D_3-1-α-hydroxylase (Murayama *et al.* 1999). Towers *et al.* (1998) proposed the 'two-step model', in which D3-bound VDR competes to bind the composite DNA site that may be recognized by VDR as well as a transcription factor, NFAT1. In the current study, however, the inhibition of transactivation by CDCA/FXR did not require VDR-DBD, which is essential for DNA-binding (Fig. 3C). Hence, competition between FXR and VDR for binding FXRE is unlikely. This may also exclude the possibility that, via the classical VDRE, D3-bound VDR may induce some factor that can suppress the transactivation by CDCA/FXR. Sakuma *et al.* (2003) reported that the transactivation by PPARα and its ligand, clofibric acid, is inhibited by D3-bound VDR, and not by estrogen/estrogen receptor or dexamethasone/glucocorticoid receptor. Intriguingly, this inhibitory effect on PPARα requires only VDR-LBD, and not the DBD. D3-bound VDR may target a common cofactor for LBD of FXR and PPARα (Sakuma *et al.* 2003). Our GST pull-down assay indicated that FXR-LBD interacts with VDR, but not RARα, PPARγ2 and LXRα (Fig. 4B). We also found that TRβ1 weakly interacted with GST-FXR (Fig 4B). Because T3-bound TRβ inhibited CDCA/FXR-induced BSEP promoter significantly (Fig. 2B), or had a tendency to repress the activation of EcRE (Fig. 1B) and SHP (Fig. 2C), direct interaction of FXR with VDR and TRβ1 may have a role in the transcriptional inhibition.

D3 has a close relationship with bile acid with respect to its biosynthesis and signal-transduction property. Both bile acid and 7-dehydrocholesterol, a precursor of D3, are

synthesized from cholesterol in liver and skin respectively. Vitamin D is hydroxylated by CYP27 in the liver to form 25-hydroxyvitamin D; CYP27 also hydroxylates bile acids and cholesterol. Here, we have demonstrated that D3-bound VDR interferes with the function of FXR in the BSEP, I-BABP and SHP promoter. Thus, D3 and VDR have a profound effect on the signal transduction mediated by bile acid/FXR. Since the reduction in the expression of BSEP and I-BABP may inhibit the enterohepatic circulation of bile acid, D3 may affect the intestinal absorption of D3 itself (Arnaud *et al.* 1975) as well as lipophilic molecules, including T_3/T_4 (DiStefano *et al.* 1993), RA (Thompson & Strautnieks 2001), TZD (Kawai *et al.* 2000), and 22(*R*)-HC. Our results also suggest that D3/VDR downregulates SHP expression (Fig. 2C). This may release the suppression of CYP7A1 gene, resulting in the consumption of cholesterol from which D3 is synthesized. The association with BsmI polymorphism was reported in primary biliary cirrhosis (PBC) (Halmos *et al.* 2000, Vogel *et al.* 2002). This polymorphism correlates with the length of singlet (A) repeat in the 3′ untranslated region of the VDR gene and affects the expression level of its mRNA (Whitfield *et al.* 2001). Although it has been postulated that D3 may modulate the immune response in this disease, our observations raise the additional possibility that the production and metabolism of bile acids may be modified by the alteration of the expression level of VDR. To avoid the complication through the possible autoregulatory mechanisms, we employed HepG2 and Caco-2 cells, but not individual animals. However, in future, intensive investigation should be performed with the VDR-null mouse (Sutton & MacDonald 2003).

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