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In vitro farnesoid X receptor ligand sensor assay using surface plasmon resonance and based on ligand-induced coactivator association

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Abstract

Ligand binding to nuclear receptors leads to a conformational change that increases the affinity of the receptors to coactivator proteins. We have developed a ligand sensor assay for farnesoid X receptor (FXR) in which the receptor–coactivator interaction can be directly monitored using surface plasmon resonance biosensor technology. A 25-mer peptide from coactivator SRC1 containing the LXXLL nuclear receptor interaction motif was immobilized on the surface of a BIAcore sensor chip. Injection of the FXR ligand binding domain (FXRLBD) with or without the most potent natural ligand, chenodeoxycholic acid (CDCA), over the surface of the chip resulted in a ligand- and LXXLL motif-dependent interaction. Kinetic analysis revealed that CDCA and its conjugates decreased the equilibrium dissociation constant (K_d) by 8–11-fold, indicating an increased affinity. Using this technique, we found that a synthetic bile acid sulfonate, 3α,7α-dihydroxy-5β-cholane-24-sulfonate, which was inactive in a FXR response element-driven luciferase assay using CV-1 cells, caused the most potent interaction, comparable to the reaction produced by CDCA. This method provides a rapid and reliable in vitro ligand assay for FXR. This kinetic analysis-featured technique may be applicable to mechanistic studies.

Keywords: Farnesoid X receptor; Ligand binding; Coactivator recruitment; Surface plasmon resonance

1. Introduction

Farnesoid X receptor (FXR) is a nuclear receptor that functions as a heterodimer with the retinoid X receptor (RXR) [1–4]. FXR is activated by natural ligand bile acids [5–7] resulting in the down-regulation of cholesterol 7α-hydroxylase, the rate-limiting enzyme of the classic pathway for bile acid synthesis in the liver [5,8]. The conversion of cholesterol to bile acid is the major pathway for eliminating cholesterol from the body [9,10]. Thus, FXR is thought to play a key role in cholesterol and bile acid homeostasis and ligands that manipulate FXR activity is expected to offer novel therapeutic strategies for lipid metabolism disorders.

The classic radio-ligand binding assay, which detects direct interactions between ligands and receptors, is commonly used for the in vitro screening of nuclear receptor (NR) ligands [11,12]. While a broad range of natural molecules are candidate ligands for NRs, the classic binding assay requires the use of specific radio-labeled ligands with a high affinity. To overcome this limitation, the coactivator recruitment assay has been developed [13]. Ligand binding to a NR leads to a conformational change, resulting in an increased affinity to coactivator proteins, such as SRC1 [14,15]. The formation of this multimeric complex augments target gene transcription. Ligand-dependent coactivator recruitment has been detected using an electrophoresis mobility shift assay [13] and has been measured using fluorescence methods, such as fluorescence resonance energy transfer (FRET) [5,6,16] or fluorescence polarization [17]. In addition, a mammalian two-hybrid assay was utilized to assess ligand-dependent FXR/coactivator interaction within

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intact cells [18]. However, these techniques that have been used for in vitro assay are limited by their requirements for radio isotopes or interference with fluorescent compounds.

In the present study, we employed a surface plasmon resonance (SPR) biosensor to directly detect receptor–coactivator interactions induced by ligands. This technique has been used in the studies for interaction between several nuclear receptors, including estrogen receptors and thyroid receptors, and coactivators [19–21]. With this system, the macromolecular interactions on the surface of the sensor chip can be quantitatively detected as changes in the refractive index. The real-time monitoring in this technique also permits the rapid analysis of kinetic parameters in a single experiment [22].

2. Materials and methods

2.1. Materials

Biotinylated peptides (>95% pure) were purchased from Biologica (Nagoya, Japan). 3α,7α-Dihydroxy-5β-cholane-24-sulfonate (CDC-sul) was synthesized as previously described [23].

2.2. Preparation of FXR ligand binding domain

DNA encoding the human FXR ligand binding domain (FXRLBD) (244 to 472; GenBank U68233) was inserted into the pGEX-6P-3 expression vector (Pharmacia Biotech, Uppsala, Sweden). GST-fused FXRLBD protein was expressed in BL21(DE3) pLysS *Escherichia coli* cells (Takara Shuzo, Shiga, Japan) and purified on Glutathione Sepharose 4B. After cleaving with PreScission protease (Amersham), the FXRLBD protein was purified on glutathione beads and dialyzed against the SPR running buffer, described below.

2.3. Surface plasmon resonance (SPR) assay

All measurements were performed using a BIAcore 3000 (BIAcore AB, Uppsala, Sweden), as described earlier [22], operating at 25 °C in a running buffer consisting of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20, and 0.5% DMSO. Streptoavidin chips (BIAcore AB) were first treated with three one-minute pulses of 50 mM NaOH containing 1 M NaCl at a flow rate of 10 μl/min. Wild-type peptide from human SRC1 (wtSRC1, CPSSHSSLTARHKILHRLLQEGSPS-CONH2, residues 676–700) [24] containing the consensus nuclear receptor interaction motif (LXXLL) [25] and the consensus-mutated SRC1 peptide (mSRC1, CPSSHSSLTARHKIAHRALQEGSPS-CONH2) were biotinylated and immobilized on individual surfaces to 122 resonance units (RU) and 124 RU, respectively. FXRLBD was preincubated with the ligand at room temperature for one hour and then injected over the surfaces at a flow rate of 20 μl/min. The ligands were dissolved in DMSO, and the final DMSO concentration was adjusted to 0.5%. After the injection was completed (120 s), the formed complex was washed with buffer for an additional 120 s. The chip surfaces were regenerated down to the peptide level by the subsequent application of a thirty-second pulse of 0.1% SDS and 10 mM NaOH.

The association rate constant (k_a) and the dissociation rate constant (k_d) of SRC1 for FXRLBD were determined using non-linear regression analysis for the initial parts of the association and dissociation phases on the sensorgram, respectively, using PRISM software (Graph Pad Software, San Diego, USA). Briefly, the initial part of the association phase was subjected to non-linear regression analysis, based on the assumption that the association would conform to the one-phase exponential association model expressed by the following equation:

$$[\text{Response}] = [\text{Plateau}] + [\text{Span}](1 - \exp(-K(t - t_0)))$$

where [Plateau] was the base line level before association, [Span] was the level of the steady-state response, K (s^{-1}) was the apparent association rate constant, t was time, and t_0 was the time at the start of the association reaction [26]. The concentration of the free FXRLBD was assumed to be constant and the same as the total FXRLBD concentration since an excess amount of FXRLBD was applied to the system, compared to the amount of immobilized SRC1 peptide. Therefore, the apparent association rate constant K was equal to $k_aC + k_d$, where C was the total FXRLBD concentration in the system and the k_a value was obtained from the equation $k_a = (K - k_d)/C$ [26]. To determine the k_d value, the concentration of free FXRLBD was rapidly reduced to 0. Then, the dissociation phase in the sensogram was subjected to non-linear regression analysis, assuming that the dissociation could be fitted to the one-phase exponential decay model expressed by the equation:

$$[\text{Response}] = [\text{Bottom}] + ([\text{Plateau}] - [\text{Bottom}])\exp(-k_d(t - t_0))$$

where [Plateau] and [Bottom] were the maximum and minimum responses for each curve, respectively, and t_0 was the time at the start of the dissociation reaction. The k_d value of the best-fitted curve was chosen for calculating the k_a and equilibrium dissociation constant K_d ($= k_d/k_a$). The k_a and k_d values were also calculated by plotting the K values against the C values, as the slope and intercept of the regression line for these plots represent the association and dissociation rate constants, respectively. Data are presented as the mean ± S.D.

2.4. Luciferase reporter assay

The monkey kidney cell line, CV-1 cells, were obtained from JCRB Cell Bank (Tokyo, Japan) and maintained in DMEM containing 10% fetal calf serum. The cells were

co-transfected with 187.5 ng of luciferase reporter plasmid (pFXRE-tk-Luc) containing four copies of the FXR response element from the phospholipid transfer protein promoter [27] upstream of the thymidine kinase (tk) promoter, 62.5 ng each of the expression vectors for FXR (pcDNA3.1-FXR) and RXRα (pcDNA3.1-RXRα), and 187.5 ng of pSV-β-Galactosidase control vector (Promega) by PolyFect (Qiagen) in 24-well plates. Three hours after transfection, the cells were treated with 20 μM of bile acids for 24 h. Cell extracts were prepared using a cell lysis buffer (Promega), and the activities of luciferase and β-galactosidase were determined. The luciferase activity was normalized to that of the β-galactosidase in each sample.

3. Results

First, we examined whether ligand binding to FXR could increase the interaction of the receptor with SRC1. A 20-mer peptide containing the LXXLL nuclear receptor interaction consensus motif [25] from SRC1 (wtSRC1) was immobilized on the sensor chip surface. FXRLBD was injected over the surface, causing a small response (sensorgram 2, Fig. 1A). The response was markedly enhanced by preincubating the FXRLBD with chenodeoxycholic acid (CDCA), a known FXR agonist (sensorgram 1). In a control study, we used a SRC1 peptide (mSRC1) containing alanine substitutions in the core motif (AXXAL). The signal generated by non-liganded FXRLBD on this mutant peptide surface was markedly lower than that produced by the wtSRC1 peptide (sensorgrams 2 versus 4), and no enhancement was observed after preincubation with CDCA (sensorgrams 3 versus 4). Thus, this data demonstrates that the ligand induced the association of FXRLBD and the SRC1 peptide in a manner that was strictly dependent on the LXXLL motif. To obtain sensorgrams showing the LXXLL motif-specific interaction, we subtracted the sensorgrams produced by the mutated peptide (3 and 4) from those produced by the wild-type peptide (1 and 2). The resulting sensorgrams (1 and 2 in Fig. 1B) clearly showed that the specific binding of the liganded FXRLBD to the SRC1 peptide was 80 RU, four-fold higher than that of unliganded FXRLBD. Since the BIAcore system allows the simultaneous detection of interaction events in four flow cells, the sensorgram detected for the wtSRC1-immobilized cell was routinely corrected with that from the mSRC1-cell to eliminate any possible responses caused by nonspecific interactions.

To measure ligand-dependent changes in the affinity between FXRLBD and wtSRC1, 1–4 μM of FXRLBD was preincubated with 100 μM of CDCA for 1 h at room temperature and then injected over a wtSRC1-coated surface. As shown in Fig. 2, the FXRLBD-CDCA complex bound to the SRC1 peptide in a FXRLBD- concentration dependent manner. The initial parts of the association (17–26 s) and dissociation (135–165 s) phases were subjected to an analysis of the association and dissociation rate constants, respectively. The initial parts of the association phase (Fig. 2B, solid line) were subjected to non-linear regression analysis after assuming that the phase conformed with the one-phase exponential association model. The value of the apparent association rate constant (K) calculated by fitting the curves for 3 μM LBD (Fig. 2B, dotted line) was $3.16 \pm 0.13 \times 10^{-1}\ s^{-1}$. The initial part of the dissociation phase (Fig. 2C, solid line) was subjected to non-linear regression analysis after assuming that the phase conformed with the one-phase exponential decay model. The k_a and K_d values were $4.98 \pm 0.14 \times 10^4\ M^{-1}\ s^{-1}$ and 3.36 ± 0.08 μM, respectively. The standard deviation of each parameter was less than 5% of the mean, indicating that the results of the assay system were highly reproducible. As another approach to determining the kinetic parameters, the K values were plotted against the injected concentrations of FXRLBD (Fig. 2D). A linear regression line of the plots

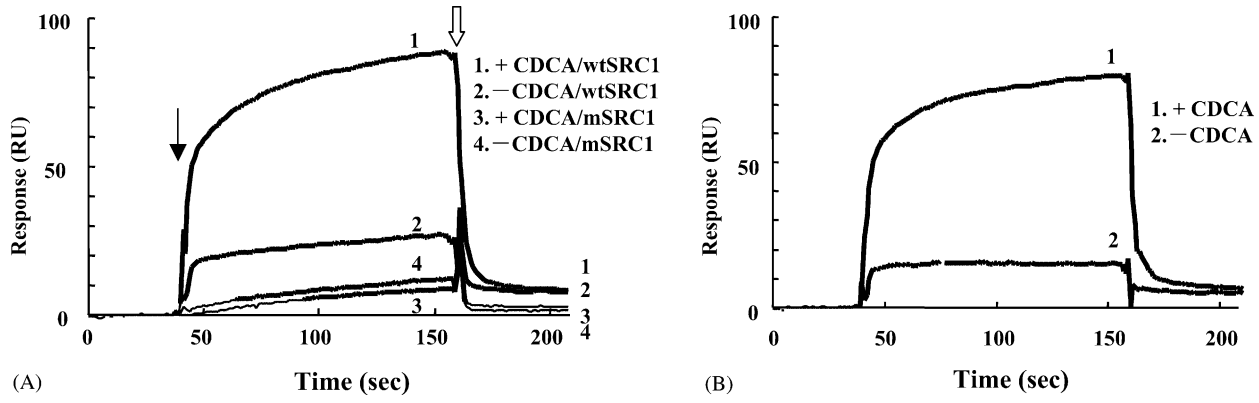

Fig. 1. Chenodeoxycholic acid (CDCA) causes a specific interaction between FXR and a SRC1 peptide containing the consensus LXXLL motif. (A) FXRLBD (0.8 μM) preincubated with or without 100 μM of CDCA was injected over surfaces containing immobilized wild-type SRC1 peptide (wtSRC1) or mutated peptide (mSRC1). The sensorgrams shown here have been corrected for bulk and background effects using the results of an unimmobilized surface. The arrow and the double arrow indicate the beginning and end points of the injections, respectively. (B) Subtraction of the sensorgrams produced by the mutated peptide (3 and 4 in Fig. 1A) from those produced by the wild-type peptide (1 and 2, respectively), results in sensorgrams that depict the consensus-specific interaction of FXRLBD with the SRC1 peptide in the presence (curve 1) or absence (curve 2) of chenodeoxycholic acid. RU, resonance units.

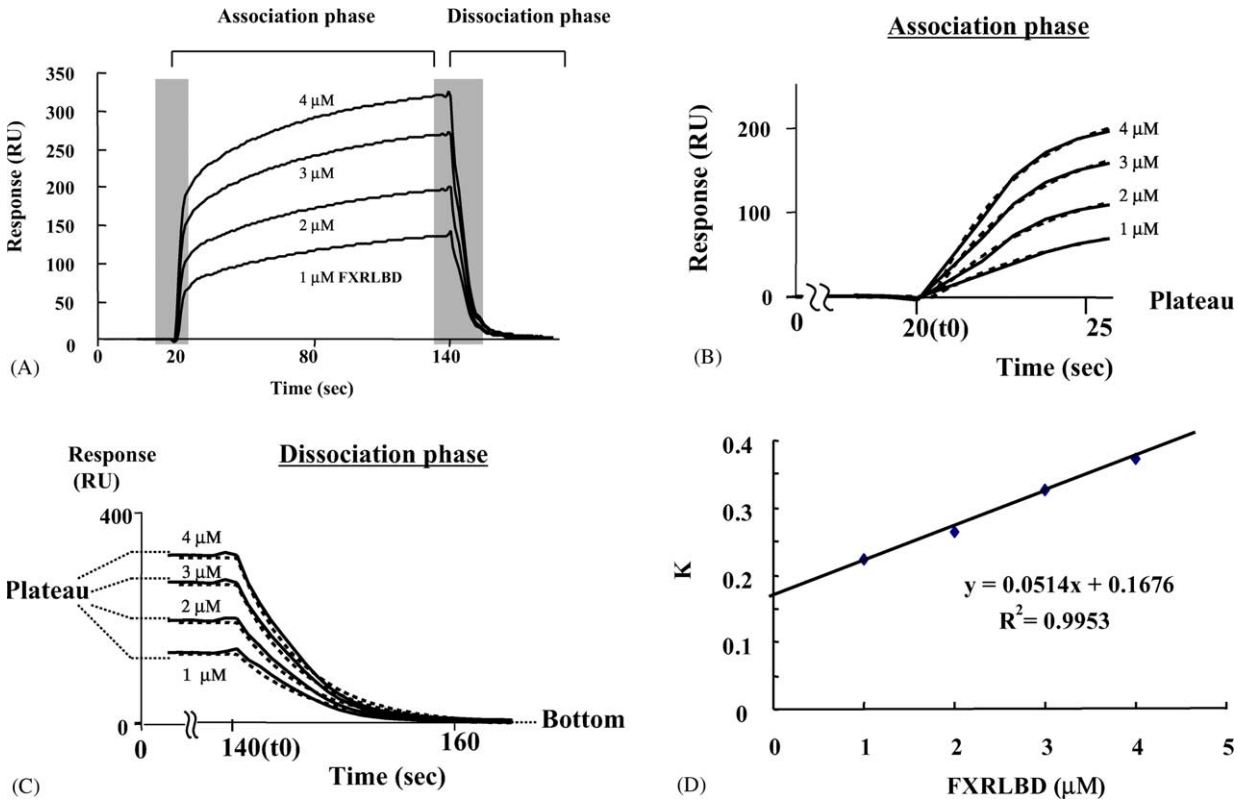

Fig. 2. Kinetic analysis of CDCA-liganded FXRLBD binding to SRC1 peptide. (A) Overlaid sensorgrams showing the injections of 1–4 μM of FXRLBD preincubated with 100 μM CDCA over a surface immobilized with wtSRC1. The sections in the gray zones were used to analyze the association and dissociation rate constants. (B) The non-linear regression analysis assumed that the association phase (solid line) conformed to the one-phase exponential association model (dotted line). (C) The non-linear regression analysis assumed that the dissociation phase (solid line) conformed to the one-phase exponential decay model (dotted line). (D) The values of the apparent association rate constants (K) were plotted against the concentrations of the injected FXRLBD (C). The rate constants for association (k_a) and dissociation (k_d) were obtained from the equation $K = k_a C + k_d$.

indicated that the k_a (slope) and k_d (intercept) values were $5.01 \pm 0.19 \times 10^4\ M^{-1}\ s^{-1}$ and $1.70 \pm 0.03 \times 10^{-1}\ s^{-1}$, respectively. The K_d value calculated from this regression line was 3.40 ± 0.19 μM. These values were consistent with those calculated using the previous method.

We next tested the ability of the SPR assay system to distinguish several known agonists from related compounds. Consistent to previous studies [5–7], CDCA, but not its conjugates with taurine (taurochenodeoxycholic acid, TCDCA) or glycine (glycochenodeoxycholic acid, GCDCA), potently activate FXR in a transient transfection assay using FXR response element-driven luciferase construct (Table 1). In contrast, As shown in Fig. 3, the interaction of FXRLBD with the SRC1 peptide was greatly enhanced with TCDCA or GCDCA. The kinetic analysis showed that the conjugates of CDCA increased the k_a values by 7–8-fold, while the k_d

Table 1
Affinity, rate constants for FXR/SRC1 interactions induced by various bile acids and their effective concentrations determined by FRET assay or cell-based luciferase assay

	k_a ($M^{-1}\ s^{-1}$)	k_d (s^{-1})	K_d (μM)	EC50 (μM) from literature[a] (FRET assay)	Relative luciferase activity[b]
No ligand	0.58×10^4	2.10×10^{-1}	36.2		1.0
CDCA	5.01×10^4	1.70×10^{-1}	3.40	4.5	18.7 ± 3.56
TCDCA	4.18×10^4	2.00×10^{-1}	4.78	10	1.13 ± 0.07
GCDCA	4.33×10^4	1.90×10^{-1}	4.39	10	1.28 ± 0.11
CDC-sul	4.60×10^4	1.78×10^{-1}	3.93		0.83 ± 0.09
UDCA	1.05×10^4	3.30×10^{-1}	31.4	ND	1.45 ± 0.10
CA	1.11×10^4	3.70×10^{-1}	33.3	ND	1.40 ± 0.46
LCA	0.93×10^4	3.10×10^{-1}	33.3	ND	4.47 ± 0.43

ND: not detected.
[a] See Parks et al. (1999) [6].
[b] Relative luciferase activity determined in the presence of 20 μM bile acids by cell-based reporter assay.

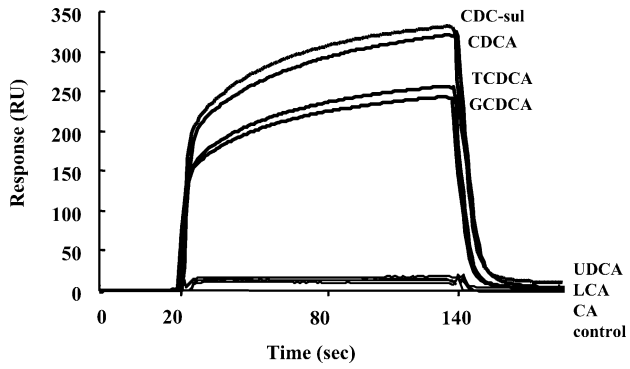

Fig. 3. FXR/SRC1 interactions caused by various bile acids. FXRLBD (4 μM) preincubated with bile acids (100 μM) was injected over the surface immobilized with wtSRC1. The sensorgrams shown have been corrected for non-specific binding using mSRC1 data.

values were marginally affected (Table 1). As reported earlier [5–7], lithocholic acid (LCA) exhibited a modest activity, whereas urosodeoxycholic acid (UDCA) and cholic acid (CA) were inactive in cell-based luciferase assay (Table 1). In this SPR assay, UDCA, CA, and LCA had little effect on the interactions (Fig. 3). Although the k_a values were slightly increased by these bile acids, increases in the k_d values resulted in an unaltered affinity. These observations were well correlated with the previously reported data obtained using a FRET assay [6] (Table 1).

Using this SPR assay, we evaluated the ability of a synthetic sulfonate-derivative of CDCA (CDC-sul) to promote SRC1 recruitment to FXR. We found that this compound, as well as the CDCA-conjugates, did not transactivate FXR in a cell-based luciferase assay (Table 1). However, this compound was shown to increase FXR/SRC1 interaction (Fig. 3) and decrease the K_d value (Table 1) as potently as CDCA in the SPR system.

4. Discussion

The luciferase reporter assay is widely used to evaluate potential nuclear receptor ligands. To employ this method for FXR ligand screening, the transfection of expression vectors encoding FXR and RXR as well as the luciferase reporter vector containing the FXRE sequence in the upper site of the luciferase coding region is necessary; the cellular luciferase activity must then be measured for use as an index of ligand transactivation. Although this method has the advantage of being able to monitor the transactivation almost in vivo, the luciferase activity may not necessarily reflect the direct binding of the ligand to the receptor, as significant transactivation by metabolites of the ligand cannot be ruled out. Moreover, the efficiency of ligand introduction into the cell is thought to vary because of differences in the charge and conformation of the ligand and the transporter expression level. Indeed, several endogenous ligands for FXR, including the most potent activator, CDCA, are able to increase luciferase expression even in non-hepatic CV-1 cells [5–7], possibly because they penetrate the cells by passive diffusion. In contrast, cholic acid or conjugated CDCA requires the coexpression of bile acid transporters, such as the sodium taurocholate cotransporter (NTCP) or the ileal bile acid transporter (IBAT), for transactivation of the FXR reporter gene in non-hepatic cells [6,7].

The in vitro radio-ligand assay is another classical method for detecting direct interactions between ligands and their receptors; in this assay, neither metabolism nor cell permeability affect the results. However, the affinity of known natural ligands for FXR is unlikely to be sufficiently high to distinguish their specific activity from nonspecific binding, as the half-maximal effective concentration (EC50) of the most potent CDCA has been shown to be 10–20 μM in cell-based or in vitro assays [5,6]. Thus, coactivator-recruitment assays using gel-shift [7], FRET [5,6], or fluorescence polarization [28] have been employed to evaluate potential FXR ligands in vitro.

In this study, we utilized SPR biosensor technology to directly and quantitatively detect ligand-dependent FXR/coactivator interactions. Real-time monitoring of interactions in this system allows kinetic parameters (k_a, k_d, and K_d) to be determined using a single injection. This advantage is a remarkable feature of this technique, since independent experiments are required for the measurement of each kinetic parameter in the classic ligand-binding assay.

In our assay system, interactions between FXRLBD and the SRC1 peptide immobilized on the surface of the biosensor chip were successfully detected as changes in the refractive index. The sensorgrams in Fig. 3 and the kinetic rate constants in Table 1 show that CDCA and its conjugates greatly increased the affinity between the receptor and the SRC1 peptide. In contrast, UDCA, CA, and LCA had marginal effects. The inactivity of CA in coactivator-recruitment has also been shown using the FRET detection system [5,6], but this finding is inconsistent with the ability of CA to transactivate FXR in the lucifease assay using transporter-expressed cells [6,7]. Our findings, together with those of previous studies [5–7], support the hypothesis that the activation of FXR by CA requires an unknown factor other than SRC1. We expect that our SPR system may serve as a useful tool for identifying this unknown factor. Other possibilities exit that CA binding to FXR requires relevant RXR-FXR heterodimer, or that CA is converted intracellularly to a potent FXR agonist.

CDC-sul is a synthetic, non-hydrolyzable model of taurine-conjugated CDCA [23] that competes with the re-absorption of bile acid from the intestine [29] and exerts a hypocholesterolemic effect [30]. Our SPR assay showed that this compound caused a strong response, comparable to that of CDCA, but did not activate FXR in a cell-based luciferase assay, probably because it cannot permeate cell membranes (Table 1). This compound has been shown to cause a down-regulation of cholesterol 7α-hydroxylase [29]. Our findings in this study indicate that this effect is mediated through FXR activation.

In conclusion, we have established an in vitro ligand sensor assay for FXR using SPR. This convenient method for performing kinetic analysis provides unique opportunities for ligand screening and mechanistic studies.

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