The Amino Acid Residues Asparagine 354 and Isoleucine 372 of Human Farnesoid X Receptor Confer the Receptor with High Sensitivity to Chenodeoxycholate*

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The critical steps in bile acid metabolism have remarkable differences between humans and mice. It is known that human cholesterol 7α-hydroxylase, the enzyme catalyzing the rate-limiting step of bile acid synthesis, is more sensitive to bile acid suppression. In addition, hepatic bile acid export in humans is more dependent on the bile salt export pump (BSEP). To explore the molecular basis for these species differences, we analyzed the function of the ligand-binding domain (LBD) of human and murine farnesoid X receptor (FXR), a nuclear receptor for bile acids. We observed a strong interspecies difference in bile acid-mediated FXR function; in the coactivator association assay, chenodeoxycholate (CDCA) activated human FXR-LBD with 10-fold higher affinity and 3-fold higher maximum response than murine FXR-LBD. Consistently, in HepG2 cells human FXR-LBD increased reporter expression more robustly in the presence of CDCA. The basis for these differences was investigated by preparing chimeric receptors and by site-directed mutagenesis. Remarkably, the double replacements of Lys366 and Val384 in murine FXR (corresponding to Asn354 and Ile372 in human FXR) with Asn406 and Ile404 explained the difference in both potency and maximum activation; compared with the wild-type murine FXR-LBD, the double mutant gained 8-fold affinity and more than 250% maximum response to CDCA in vitro. This mutant also increased reporter expression to an extent comparable with that of human FXR-LBD in HepG2 cells. These results demonstrate that Asn354 and Ile372 are critically important for FXR function and that murine FXR can be "humanized" by substituting with the two corresponding residues of human FXR. Consistent with the difference in FXR-LBD transactivation, CDCA induced endogenous expression of human BSEP by 10–12-fold and murine BSEP by 2–3-fold in primary hepatocytes. This study not only provides the identification of critical residues for FXR function but may also explain the species difference in bile acids/cholesterol metabolism.

Bile acids are synthesized from cholesterol in the liver (1). Bile acids not only provide the detergent function needed to solubilize vitamins and fats, but they also act as signal molecules in controlling a wide array of biological processes (2). Although bile acid signaling pathways are preserved in mammals, it is known that these pathways have profound species differences. It is shown that expression of cholesterol 7α-hydroxylase (Cyp 7a),1 the enzyme catalyzing the first and rate-limiting step of bile acid synthesis (3), is highly variable in different species (4). Human Cyp 7a has a lower level of basal expression and is more sensitive to bile acid inhibition than murine Cyp 7a (4–6). Cholesterol feeding increases Cyp 7a expression in mice but not in humans, and the feedforward mechanism in mice was proposed to be mediated through an LXRE present in the murine Cyp 7a promoter (7, 8). In addition, bile salt export pump (BSEP)-mediated bile salt secretion, another critical step in bile acid metabolism, has remarkable species differences (9). Deficiency of BSEP in men results in progressive familial intrahepatic cholestasis type 2 (10), a severe liver disease that impairs bile flow and causes irreversible liver damage. Patients with this disease secrete less than 1% of biliary bile salts compared with normal individuals (11). In contrast, BSEP-null mice secrete 30% bile salts compared with wild-type mice, have unimpaired bile flow, and do not develop cholestasis even though the bile acid concentration in bile is decreased dramatically (12). Currently, the molecular basis for the above species differences is unclear.

Farnesoid X receptor (FXR) is a nuclear receptor for bile acids (13–15). Bile acids such as chenodeoxycholate (CDCA), deoxycholate, cholate, and lithocholate are each a ligand of FXR. CDCA is the most potent endogenous agonist (13, 14). CDCA binding to the ligand-binding domain (LBD) of FXR causes a receptor conformational change and recruitment of coactivators such as steroid receptor coactivator protein-1 (SRC-1), which in turn results in activation of transcription (14). FXR regulates transcription of genes to allow feedback control of bile acid synthesis and secretion (13, 14). Many, if not all, bile acid-modulated biological processes have been demonstrated to be mediated through FXR. It has been shown that FXR inhibits expression of Cyp 7a (16–19) and activates expression of intestinal bile acid-binding protein (20), phospholipid transfer protein (21), BSEP (22), and apoC-II (23) and apoA-I (24).

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The abbreviations used are: Cyp 7a, cholesterol 7α-hydroxylase; BSEP, bile salt export pump; CDCA, chenodeoxycholate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid CMV, cyto-megalovirus; CS-FBS, charcoal-stripped fetal bovine serum; DBD, the DNA-binding domain; DMEM, Dulbecco’s modified Eagle’s medium; FXR, farnesoid X receptor; GST, glutathione S-transferase; LBD, the ligand-binding domain; LXRE, liver X receptor responsive element; LUC, luciferase; PPAR, peroxisome proliferator-activated receptor γ; RXR, retinoid X receptor α; SRC-1, steroid receptor coactivator protein-1; th, thymidine kinase; LXR, liver X receptor; LXXRE, liver X receptor responsive element.

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The nuclear receptor LBD consists of 12 helices (25, 26). Previous studies suggest that amino acid residues in helices 3, 4, 5, and 12 play important roles for interaction with coactivators (27). However, it was not known that amino acids in helices 7 and 8 also have a critical role for nuclear receptor function. In this study, we first observed species differences in the function of murine and human FXR-LBD, and we then identified the critical residues responsible for the difference. We demonstrate that Asn^{354} and Ile^{372} in helices 7 and 8 of human FXR-LBD confer on the receptor a robust response to CDCA. Murine FXR-LBD with Lys and Val at these two positions was less robustly activated by CDCA compared with murine BSEP in primary hepatocytes. This study provides the identification of critical residues for FXR function may also explain the species difference in bile acids/cholesterol metabolisms.

MATERIALS AND METHODS

Reagents—The following reagents were obtained from Invitrogen: DMEM and Opti-MEM I, regular and charcoal-stripped fetal bovine serum (CS-FBS), TRIZOL reagents, PCR Supermix, and oligonucleotide primers. FuGENE 6 transfection reagent was obtained from Roche Diagnostic Corp. Reagents for the β-galactosidase and luciferase assays were purchased from Promega (Madison, WI). CDCA was obtained from Steraloids, Inc. (Newport, RI). The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). The nuclear receptor LBD consists of 12 helices (25, 26). Prior studies suggest that amino acid residues in helices 3, 4, 5, and 12 play important roles for interaction with coactivators (27). However, it was not known that amino acids in helices 7 and 8 also have a critical role for nuclear receptor function. We demonstrate that Asn^{354} and Ile^{372} in helices 7 and 8 of human FXR-LBD confer on the receptor a robust response to CDCA. Murine FXR-LBD with Lys and Val at these two positions was less robustly activated by CDCA compared with murine BSEP in primary hepatocytes. This study provides the identification of critical residues for FXR function may also explain the species difference in bile acids/cholesterol metabolisms.

Site-directed Mutagenesis—All mutants were made on pGEX-KG or pcDNA3.1-Gal4-FXR-LBD using the QuikChange site-directed mutagenesis kit according to the manufacturer’s instructions. The integrity of the sequence was confirmed by DNA sequencing.

Preparation of GST-FXR-LBD Fusion Proteins—Escherichia coli strain BL21 harboring pGST-hFXR-LBD or pGST-mFXR-LBD or various mutations was cultured in LB medium to a density of OD_{600} 0.7–1.0 and induced for overexpression by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.2 mM. The isopropyl-1-thio-β-D-galactopyranoside-induced cultures were grown at 25 °C for an additional 4 h. The cells were then harvested and lysed by sonication for purification of GST-fusion proteins according to the recommended procedure from Amersham Biosciences using glutathione-Sepharose beads. The purification of all of the GST fusion proteins that were used in this study did not involve denaturation/renaturation steps.

FXR Coactivator Association Assays—A homogeneous time-resolved fluorescence-based FXR and coactivator SRC-1 interaction assay was used to examine the interaction of FXR-LBD with various ligands according to methods described previously for other nuclear receptors (28) with minor modifications. Briefly, 198 μl of reaction mixture (100 mM HEPES, 125 mM KF, 0.125% (w/v) CHAPS, 0.05% dry milk, 4 mM GST-FXR-LBD (human, murine, or mutant), 2 μM oleosin, 10 μM SRC-1 fragments, 0.1 μM human SRC-1, and 200 nM NSP8RN1Qp to VKVKEVKKQK; murine SRC-1, amino acids NSP8RSLSMQP to VVKVEKKQK) 20 nM SAXL665 (streptavidin-labeled allophycocyanin) were added to each well, followed by 2 μl of dimethyl sulfoxide or various concentrations of CDCA into appropriate wells. Plates were incubated overnight at 4 °C, followed by measurement of fluorescence on a Packard Biotek Microplate Reader (model 2000), with excitation at 480 nm and emission at 520 nm. The CDCA response, expressed as the ratio of the emission intensity at 665 nm to that at 620 nm, was multiplied by a factor of 10.

Nuclear Extraction and Western Blot Analysis for Expression of Gal4-FXR-LBD—HeP2 cells, a human hepatoma cell line obtained from ATCC, were maintained in DMEM containing 10% FBS, 1% penicillin/streptomycin, and 1 mM sodium pyruvate. Cells were seeded at a density of 4 × 10^{4} cells/plate of 10-cm plates in DMEM 24 h before transfection. Cells were transfected with transfection mixes in serum-free Opti-MEM I using FuGENE 6 transfection reagent according to the manufacturer’s instructions. Typically, transfection mixes for each plate contained 18 μl of FuGENE 6, 3 μg of pcDNA3.1-Gal4-FXR-LBD (human, murine, or mutant) expression vector, and 3 μg of pcDNA3.1-hRXRα expression construct. Cells were then incubated for 4 h at 37 °C in an atmosphere of 10% CO_{2}. The cells were then incubated for 40–48 h in fresh DMEM containing 5% CS-FBS with 25 μM CDCA. At the end of the incubation, nuclear extraction was prepared using a Nuclear and Cytoplasmic Extraction kit (Pierce Chemical Co.) according to the manufacturer’s instructions. Typically, 30 μg of total nuclear protein was separated by electrophoresis on a 4–20% SDS-PAGE (Invitrogen). Western blotting was carried out following the manufacturer’s instructions (Amersham Biosciences) using the polyclonal rabbit anti-Gal4-DDD antibody (Upstate Biotechnology, Lake Placid, NY). Donkey anti-rabbit IgG conjugated to horseradish peroxidase and the ECL chemiluminescence kit were purchased from Amersham Biosciences.

Gal4 FXR-LBD Transactivation—HeP2 cells were seeded at a density of 3.2 × 10^{4} cells/well of 96-well plates 24 h before transfection. Cells were transfected with transfection mixes in serum-free Opti-MEM I using FuGENE 6 transfection reagent as described above. Transfection mixes for each well contained 0.405 μl of FuGENE 6, 3 ng of pcDNA3.1-Gal4-FXR-LBD (human, murine, or mutant) expression vector, 3 μg of pcDNA3.1-hRXRα expression construct, 60 ng of pUAS/SX1 (Amersham Biosciences), and 2 μg of pmCV-lacZ. Cells were then incubated for 4 h at 37 °C in an atmosphere of 10% CO_{2}. The cells were then incubated for 40–48 h in a fresh DMEM containing 5% CS-FBS with or without various concentrations of ligands. Cell lysates were produced using reporter lysis buffer according to the manufacturer’s directions. Luciferase and β-galactosidase activities in cell extracts were determined as described previously (29). Luciferase activities were normalized to β-galactosidase activities individually for each cell.

Primary Human and Murine Hepatocytes—Plated primary human hepatocytes were obtained from In Vitro Technologies (Baltimore, MD). Plated murine hepatocytes were prepared according to the protocol described previously (30). Cells were seeded at a density of 2 × 10^{4} cells/well of six-well plates in DMEM containing 10% FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 25 μg/mL HEPES and cultured at 37 °C in an atmosphere of 5% CO_{2} for 24 h. Cells were then incubated with various concentrations of CDCA in phenol red-free DMEM containing 0.5% CS-FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, 2 mM t-glutamine, and 25 μg/mL HEPES for 24 h.

RNA Isolation and Real-time Quantitative PCR—Total RNA was extracted from the cultured cells using the TRIZOL reagent according to the manufacturer’s instructions. Reverse transcription reactions and TaqMan–PCRs were performed according to the manufacturer’s instructions (Applied Biosystems). Sequence-specific amplification was detected with an increased fluorescent signal of FAM (reporter dye) during the amplification cycles. Amplification of human 18 S RNA was used in the same reaction of all samples as an internal control. Gene-specific primers were designed using Primer Express software and were synthesized by Applied Biosystems. These sequences (5’ to 3’) are as follows:
transfected with 0.405 cells were treated with various concentrations of CDCA for 40 s instructions. The transfected reagent according to the manufacturer’s version vector in serum-free Opti-MEM I using FuGENE 6 transfection pcDNA3.1-Gal4-mFXR-LBD (Gal4-mFXR-LBD, striped bars) or pcDNA3.1-Gal4-hFXR-LBD (Gal4-hFXR-LBD, open bars) expression vector in serum-free Opti-MEM I using FuGENE 6 transfection reagent according to the manufacturer’s instructions. The transfected cells were treated with various concentrations of CDCA for 40–48 h, and the cell lysate was used for determination of luciferase and β-galactosidase activities as described under “Materials and Methods.” Luciferase activities were normalized to β-galactosidase activities individually for each well. Each value represents the mean ± S.D. of six determinations.

The human BSEP forward primer was GGGCCATTGTACCAGAGACTCTAA. The probe was 6FAM-TCTTGTACTAGATTGGCAGCCTTAGA-TAMRA. The reverse primer was TTCCAGTTAAAGAGGA-

The human SRC-1 protein was used in the coactivator association assay. To eliminate the possibility that the low functionality observed for murine FXR-LBD is more sensitive to CDCA, resulting in high functionality compared with murine FXR-LBD.

The human SRC-1 protein was used in the coactivator association assay. This assay measures CDCA-induced interaction between FXR-LBD and the coactivator SRC-1 in a cell-free environment. This interaction often reflects the transactivation ability of nuclear receptors in vivo (28). Similar to the transactivation in HepG2 cells, the murine FXR-LBD displayed a 30–40% maximal response to CDCA compared with human FXR-LBD (Fig. 2A). In addition, the half-maximal stimulation (EC50) of CDCA on the murine receptor was 10-fold higher than that on the human receptor, with an EC50 value of 49.8 μM and 5.2 μM on the two receptors, respectively (Fig. 2A). This result demonstrates again that human FXR-LBD is more sensitive to CDCA, resulting in high functionality compared with murine FXR-LBD.

The human SRC-1 protein was used in the coactivator association assay. To eliminate the possibility that the low functionality observed for murine FXR in coactivator association resulted from the use of human SRC-1, analysis was also performed in a similar assay using murine SRC-1. Nearly identical...
results were obtained from the assay using murine SCR-1. Murine FXR-LBD also showed much less robust activation by CDCA than did human FXR-LBD; the EC$_{50}$ value of CDCA on murine FXR-LBD (47.9 $\mu$M) was 8-fold higher than that on the human receptor (6.1 $\mu$M) (Fig. 2B). This result suggests that the differential response to CDCA displayed by human and murine FXR-LBD was not caused by a species difference of SRC-1, it is rather an intrinsic property of FXR.

The C Terminus of Human FXR Contains Amino Acid Residues Critically Important for Transactivation—To identify the amino acid residues critical for CDCA-mediated FXR function, two chimeric receptors were constructed between human and murine FXR-LBD using two EcoRI sites commonly occurring in both receptors (Fig. 3A). The first chimera (chi-1) consists of one-third murine and two-thirds human FXR-LBD, and the second chimera (chi-2) contains approximate two-thirds murine and one-third human FXR-LBD (Fig. 3A). In the coactivator association assay, both chi-1 and chi-2 displayed robust activation by CDCA with a maximum activation of 80 and 75% of human FXR-LBD, respectively (Fig. 3B). CDCA had an EC$_{50}$ value of 7.2 and 11.9 $\mu$M, respectively, on chi-1 and chi-2. These EC$_{50}$ values are similar to the EC$_{50}$ of 6.8 $\mu$M on the human receptor (Fig. 3B). These results suggest that the C terminus of human FXR contains residues critical for CDCA-mediated FXR function.

Identification of Asn$_{354}$ and Ile$_{372}$ as Residues Critical for FXR Function—The C-terminal fragment of human and murine FXR (residues 353–472 of human FXR) differs at four residues, at positions 354, 372, 421, and 422 (Fig. 4, according to human FXR). Each of the four residues (Asn$_{354}$, Ile$_{372}$, Ile$_{421}$, and His$_{422}$) in human FXR was introduced individually into the murine receptor (mFXR$_{366N}$-LBD, mFXR$_{384I}$-LBD, mFXR$_{433I}$-LBD, and mFXR$_{434H}$-LBD), and the mutant receptors were characterized in a coactivator association assay for the responsiveness to CDCA. Two of the single mutants, GST-mFXR$_{433I}$-LBD and GST-mFXR$_{434H}$-LBD, were indistinguishable from the wild-type murine FXR in both EC$_{50}$ and the maximal response of CDCA (Fig. 5), indicating that the two residues may not be critical for CDCA-induced activation and may not contribute significantly to the species difference in FXR function. In contrast, the other two single mutants, GST-mFXR$_{366N}$-LBD and GST-mFXR$_{384I}$-LBD, showed a significant increase in responsiveness to CDCA compared with the wild-type murine FXR-LBD, with an EC$_{50}$ of 18.4 and 37.8 $\mu$M, respectively, and a maximal response of 2–3-fold higher than murine FXR. However, none of the single mutants had activity comparable with that of the wild-type human receptor (Fig. 5).

The double substitutions (Asn$_{366}$ and Ile$_{384}$) were introduced to murine FXR to determine whether these two residues together would humanize the murine receptor. Indeed, this double mutant (GST-mFXR$_{366N}$-$384I$-LBD) showed a dramatic increase in responsiveness to CDCA compared with the wild-type murine receptor (Fig. 6). In the coactivator association assay, GST-mFXR$_{366N}$-$384I$-LBD had a maximal response of 75% that for human FXR-LBD. The EC$_{50}$ of CDCA on GST-mFXR$_{366N}$-$384I$-LBD was 9.2 $\mu$M, which was also comparable with the EC$_{50}$ value of 6.8 $\mu$M on human FXR-LBD (GST-hFXR-LBD). In both maximal stimulation and EC$_{50}$ of CDCA, GST-mFXR$_{366N}$-$384I$-LBD closely resembled chi-2, which contains four amino acid alterations in the C terminus of murine FXR-LBD (Fig. 6). These data suggest that alterations at these two positions of murine FXR account for the difference in FXR function, and that Asn$_{354}$ and Ile$_{372}$ are critically important for CDCA-mediated FXR activation function.

The important role of Asn$_{354}$ and Ile$_{372}$ in human FXR-LBD was also confirmed in Gal4-FXR transactivation in HepG2 cells. Consistent with the results in the coactivator association assay, Gal4-mFXR$_{366N}$-$384I$-LBD increased luciferase activity in HepG2 cells in a CDCA dose-dependent manner with a maximal induction of more than 250% that induced by wild-type murine Gal4-FXR-LBD (Fig. 7A). This value was approximately 75% of the maximal induction displayed by human Gal4-FXR-LBD (Fig. 7A). The receptor expression was detected by Western blotting. The expression level of Gal4-mFXR$_{366N}$-$384I$-LBD was approximately as same as that of wild-type Gal4-mFXR-LBD, and the expression of human FXR-LBD (Gal4-hFXR-LBD) was slightly lower than the murine receptor (Fig. 7B). These data confirm the critical role of Asn$_{354}$ and Ile$_{372}$ residues in FXR function and indicate that the murine receptor...
was humanized by the introduction of the two amino acid substitutions.

Induction of BSEP mRNA by CDCA in Primary Human and Murine Hepatocytes—BSEP is a direct target gene of FXR (22, 32). The induction of BSEP mRNA correlates well with FXR transactivation (32). To compare the functionality of human and murine FXR further, primary hepatocytes of humans and mice were prepared and treated with various concentrations of CDCA, and the endogenous expression of BSEP was analyzed by real time PCR (TaqMan). CDCA robustly increased BSEP mRNA in a dose-dependent manner with a maximal induction of $10^{-12}$-fold (Fig. 8) in human cells. However, CDCA only increased murine BSEP mRNA by 2–3-fold (Fig. 8). This result is consistent with the observed difference in FXR-LBD function between the two species and supports the conclusion that human FXR has high sensitivity to bile acids such as chenodeoxycholate.

**DISCUSSION**

Bile acid metabolism has profound differences between humans and mice. To seek the molecular basis for the species difference, we investigated the CDCA-mediated FXR function using human and murine FXR-LBD. We observed that human FXR-LBD was much more robustly activated by CDCA than murine FXR-LBD. In addition, the affinity of CDCA on human FXR-LBD was 10-fold higher than that on murine FXR-LBD in the coactivator association assay. These results suggest that human FXR is more sensitive and susceptible to CDCA. Indeed, in primary human hepatocytes, BSEP expression was induced more robustly by CDCA. These results provide strong evidence for explaining the species difference in bile acid/cholesterol metabolism.

Transactivation data reported by Parks et al. (14) showed a smaller difference between the full-length human and murine
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FXR, although the difference was also observed. There are three potential explanations for this discrepancy. First, the data by Parks et al. had a relatively large error bar, which may mask the real difference between human and murine FXR. Second, a high concentration of CDCA (100 μM) was used by Parks et al. CDCA is a hydrophobic bile acid and has profound ligand-dependent formation of a hydrophobic pocket for binding of coactivators (27). Prior to this study, amino acid residues in helices 3, 4, and 5 were also implicated in the association assay used in this study could not distinguish these species differences in FXR function by preparing chimeric receptors and by site-directed mutagenesis. Remarkably, two amino acid differences in helices 7 and 8 appear to explain the dramatic differences in human and murine FXR function. Substitutions of Lys → Asn and Val → Ile, respectively, at the two corresponding positions of murine FXR humanized the murine receptor. We conclude that it is Asn$^{354}$ and Ile$^{372}$ that confer human FXR with high sensitivity to CDCA.

Helix 12 (or AF-2) of nuclear receptors plays an essential role for nuclear receptor transactivation. Deletion or mutation of helix 12 completely destroyed the activation function of PPARγ (33), estrogen receptor (34), chicken thyroid hormone receptor alpha (35), thyroid hormone receptor beta (36), and RAR/RXRα (37). Residues in helices 3, 4, and 5 were also implicated in the ligand-dependent formation of a hydrophobic pocket for binding of coactivators (27). Prior to this study, amino acid residues in helices 7 and 8 had not been assigned with critical roles. This study is the first demonstration for a critical role for residues in helices 7 and 8.

Sequence alignment of human PPARγ (38), thyroid hormone receptor beta 2 (39), RARγ (40), and LXRα (41, 42) predicts Asn$^{354}$ as a potential contact site with the ligand. Replacement with Val at this position in murine FXR may therefore decrease the binding affinity for CDCA. A classical receptor-binding assay would determine whether this residue is critical for ligand binding or for coactivator recruiting. The coactivator association assay used in this study could not distinguish these two processes. Ile$^{372}$ of human FXR is conserved among several nuclear receptors (PPARγ, TRγ2, RARγ, and LXRα). Although computer modeling does not predict a critical role for this
residue, it may contribute to the formation of coactivator binding pocket with its hydrophobic side chain. Ile\textsuperscript{372} may also be involved in ligand binding through the interaction of its hydrophobic side chain with the sterol core of bile acids. In any event, evolution may select these two natural alterations in FXR to adapt specific needs in mice.

The discovery of species differences in FXR function further supports the notion of FXR as a bile acid sensor. Given the fact that human FXR is more sensitive to bile acids, it is likely that FXR plays more important roles in humans than in rodents, and FXR ligands may have potential as therapeutic drugs for intrahepatic cholestasis and lipid disorders. This study provides the first identification of critical residues in helices 7 and 8 and reinforces cautious extrapolation of ligand activity across highly conserved receptors.

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