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# The important role of residue F268 in ligand binding by LXRβ

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**Abstract Liver X receptors (LXRs) are nuclear receptors that regulate the metabolism of cholesterol and bile acids. Despite information on the specificity of their natural ligands, oxysterols, relatively little is known about the ligand binding site in LXRs. The helix 3 region in the ligand binding domain (LBD) of peroxisome proliferator-activated receptors (PPARs) has been implicated in ligand entry. Sequence alignment of LXRs, farnesoid X receptor (FXR), and PPARs identified the corresponding helix 3 region in the LXRβ LBD. Residues F268 and T272, which are conserved in all the aligned sequences and only in LXRs and FXR, respectively, were replaced with alanine. The effects of these mutations on ligand binding and receptor activation were examined using an in vitro ligand binding assay and a cell based reporter assay, respectively. The LXRβ mutant F268A did not bind ligand. In contrast, conversion of T272 to alanine has no effect on ligand binding. By transiently expressing a chimeric receptor containing *Escherichia coli* tetracycline repressor (TetR) and LXRβ LBD and a reporter with a TetR binding site, we show that mutant F268A lost the ability to activate transcription of the reporter, whereas mutant T272A still has an activity similar to that of the wild-type LXRβ. These data, consistent with the findings in the in vitro ligand binding assay and our 3D modeling, are the first study that identifies a residue critical for ligand binding in LXRβ.** 

*Key words:* Liver X receptor; Ligand binding domain; Scintillation proximity assay

## 1. Introduction

Liver X receptors (LXRs) are members of a nuclear receptor superfamily that induce ligand dependent transcriptional activation of target genes [1]. There are two subtypes of LXRs, LXRα and LXRβ. LXRα is preferentially expressed in liver, small intestine, kidney and spleen [2,3]. In contrast, LXRβ expression is ubiquitous [4]. The genomic structure and the promoter regions of the two LXR genes contain specific regulatory sites, which suggest that LXRs may have physiological roles in the immune system [5].

Like other nuclear receptors, LXRs heterodimerize with retinoid X receptor (RXR) for function [1]. However, LXRs represent a subclass of permissive nuclear receptors, including PPARs and FXR [1]. In this subclass, the RXR heterodimers can be activated independently by either the RXR ligand, the partner's ligand, or synergistically by both [1]. LXRs are activated by naturally occurring oxysterols and regulate the expression of target genes [6–8], including ATP binding cassette transporter 1 (ABC1), ATP binding cassette transporter 8 (ABC8) and cholesterol ester transfer protein (CETP) [9–11]. These findings suggest that LXRs might regulate lipoprotein metabolism, especially that of high density lipoprotein (HDL) [9–11]. In addition, LXRα increases the expression of cholesterol 7-α hydroxylase, the rate-limiting enzyme in bile acid synthesis [12]. In LXRα knockout mice, there is an accumulation of cholesterol esters and a deficiency in the conversion of cholesterol to bile acids [13], suggesting that LXRα is responsible for bile acid synthesis. In addition, LXRβ is expressed in the livers of these mice and did not compensate for the loss of LXRα, suggesting that LXRβ may have different biological functions.

The two functional domains, including the N-terminal DNA binding domain (DBD) and the C-terminal ligand binding domain (LBD), mediate the transcriptional activation function of nuclear receptors [14]. The LBD has been the focus of intense study since it determines ligand specificity [15,16]. In contrast to PPARs in the same subclass [17,18], LXR LBDs and the critical helical regions within the LBDs have not been characterized. Among the 12 helices in the LBD of PPARα, the carboxyl helices are required for heterodimerization with RXR and the N-terminal helices are involved in forming ligand binding pockets [15,16,19]. Interestingly, a single substitution of Glu282 with glycine in helix 3 of mouse PPARα altered the transcriptional response to ligands, suggesting that helix 3 is important for ligand selectivity [17]. In order to study ligand dependent activation of LXRβ, we have identified a region in LXRβ LBD that corresponds to helix 3 in PPARs. We have examined the effects of mutations within this region on ligand binding and transcriptional activation by LXRβ. Our results provide evidence for the important role of residue F268 in ligand binding by LXRβ LBD.

## 2. Materials and methods

### 2.1. Materials

[$^3$H]24(*S*)-hydroxycholesterol was synthesized by Amersham. Non-

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labeled 24(*S*)-hydroxycholesterol was synthesized by SH-ICON (Chicago, IL, USA). The His·Bind protein purification kit was from Novagen. Scintillation proximity assay (SPA) beads were from Amersham. Tissue culture media and transfection reagents were from Gibco BRL. Restriction enzymes were from New England Biolabs or Gibco BRL. Luciferase and β-galactosidase assay kits were from Promega.

### 2.2. Vector construction, mutagenesis and protein expression

Human LXRβ LBD containing amino acids 203–461 was amplified by polymerase chain reaction. The 5′ primer (CAT ATG CCT GGT GGA TCT GAG GCA GGC) contains a *Nde*I site so that after ligation, the LXRβ LBD is in-frame with the initiation methionine and the N-terminal HIS tag in the expression vector pET16b (Novagen). The 3′ primer (GGA TCC CCC TCA CTC GTG GAC GTC CCA) contains a stop codon followed by a *Bam*HI site for convenient cloning. The expression vector pET-LXRβ LBD was transformed into *Escherichia coli* BL21 (DE3). Bacteria cultures were grown at 37°C until $OC_{595}$ reached 0.5 and induced at room temperature with 50 μM IPTG for 3 h. Lysates were prepared for direct SPA based ligand binding assay [9,20] or protein purification according to the manufacturer's instructions. Mutagenesis reaction was carried out using an ExSite site directed mutagenesis kit (Stratagene). Protein purification was carried out with a His·Bind protein purification kit (Novagen) according to the manufacturer's instruction manual.

### 2.3. SPA based ligand binding assay

An SPA based ligand binding assay on a 96 well plate format was developed using [$^3$H]24(*S*)-hydroxycholesterol, His-tagged protein and copper-coated SPA beads. In each well of 96 well clear bottom plates (Costar, Cornings, NY, USA), bacterial lysates or purified protein were incubated with 200 nM [$^3$H]24(*S*)-hydroxycholesterol in a final volume of 100 μl binding buffer (50 mM Tris–HCl, 10% glycerol, 10 mM sodium molybdate) at room temperature for 30 min. Beads buffer (25% beads (v/v), 25% bovine IgG (v/v), 50% binding buffer (v/v)) was added (50 μl/well) and incubated for 30 min. The plates were set on a bench for 10 min to allow the beads to settle on the bottom of the plates before being counted on a β counter.

### 2.4. Gel electrophoresis and Western blotting

Samples were resolved electrophoretically on 4–20% gradient gels at a constant voltage (100–180 V). Proteins were transferred to Immobilon-P membranes using a semi-dry electroblotter. The membranes were washed with TBST buffer (20 mM Tris–HCl, 137 mM NaCl, 0.5% Tween-20, pH 7.6), blocked in blocking buffer (TBST containing 5% dry milk) for 1 h at room temperature, and incubated with anti-HIS monoclonal antibody (Novagen) for 1 h at room temperature. Blots were washed extensively with TBST and incubated with horseradish peroxidase conjugated secondary antibody. After 1 h, blots were washed as above and developed with an enhanced chemiluminescence kit (Amersham).

### 2.5. Transfection and reporter assay

CHO cells were maintained in MEMα media supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified 5% atmosphere. Cells were seeded on 12 well plates (Costar) with 1 ml media in each well. When reaching ~80% confluence, the cells were switched to MEMα media containing 5% lipoprotein deficient serum (LPDS) (Cocalico Biologicals) and maintained for 24 h before transfection using the lipofectamine mediated method (Gibco BRL). The cells were transfected with the following constructs: wild-type or mutant forms of TetR-LXRβ LBD (0.05 μg/well), pTet-Luc (0.2 μg/well) and pSV-β-gal (0.2 μg/well) [21]. An equal amount of DNA was used in each transfection using pcDNA3.1 as complement. Four h after incubated with the transfection mix, the cells were washed and treated with 50 μM 24(*S*)-hydroxycholesterol (dissolved in dimethyl sulfoxide, DMSO) or DMSO for 24 h in MEMα medium plus 5% LPDS. The final DMSO concentration in the medium did not exceed 0.2% (v/v). The cells were harvested with lysis buffer provided by the luciferase or β-galactosidase (β-gal) assay kit and enzyme assays were carried out according to the manufacturer's manual (Promega).

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|---|---|---|---|
| **hLXRα** | 251 | QQR**F**AHF**TE**L**A**IVS**VQ**E | 267 |
| **hLXRβ** | 265 | QQR**F**AHF**TE**L**A**IIS**VQ**E | 281 |
| **hFXR** | 281 | EEN**F**LIL**TE**M**A**TNH**VQ**V | 297 |
| **hPPARα** | 270 | VRI**F**HCCQCTSV**E**T**V**TE | 286 |
| **hPPARβ** | 243 | VHV**F**YRCQCTTVET**V**RE | 259 |
| **hPPARγ** | 279 | IRI**F**QGCQF**R**SV**E**A**V**Q**E** | 295 |

Fig. 1. Sequence alignment of helix 3 regions in the ligand entry domains from LXRs, FXR and PPARs. The residues conserved in all the nuclear receptors or only in the LXRs and FXR are highlighted. The residues important in ligand selectivity as demonstrated in previous studies are outlined. The mutated residues in LXRβ are indicated by arrows.

## 3. Results and discussion

### 3.1. Identification of amino acid residues that may be involved in ligand binding by LXRβ

Previous work on PPARs, RXRα and RARγ has identified functional helical regions that are involved in ligand binding, among which helix 12 acts as a lid to close the binding pocket and helix 3 is involved in ligand entry [14,15,22]. In PPARγ, R288, E291 and E295 in helix 3 are major determinants of the ligand entry site [22]. However, none of these amino acids are conserved in LXRs or FXR (Fig. 1), which is consistent with the fact that LXRs and FXR have ligands structurally distinct from those for PPARs [8,9,23]. In addition, the glutamic acid residue in PPARα corresponding to E291 in PPARγ has been demonstrated to be important in ligand selectivity [17] (Fig. 1). However, the glutamic acid is only conserved in PPARs in the alignment of helix 3 regions from LXRs, FXR and PPARs (Fig. 1). Despite the structural difference between their ligands, LXRs and PPARs do share conserved residues in their helix 3 regions (Fig. 1), among which a phenylalanine (F268 in LXRβ) and a valine (V279 in LXRβ) are conserved across all sequences. V279 in LXRβ corresponds to V293 in PPARγ, which is in proximity to residues R288, E291 and E295 at the carboxyl terminus of helix 3, a region important for ligand selectivity in PPARγ [22]. In contrast, F268 in LXRβ is close to the amino terminus of helix 3 and its role in ligand binding has not been defined. In addition, four residues are conserved only in LXRs and FXR, including T272, E273, A275 and Q280 in LXRβ (Fig. 1). Among these residues, T272 is the most amino terminal. To date, the importance of the carboxyl half of helix 3 in ligand entry has been established [14,15,22]. Here we made mutations at F268 and T272 to identify the roles of the conserved residues in the N-terminus of helix 3 in LXRβ.

### 3.2. Mutagenesis, expression and purification of LXRβ LBD

To identify the roles of residues F268 and T272 in oxysterol binding by LXRβ, we made alanine replacement mutations at F268 and T272 of LXRβ LBD, respectively, and examined the effects on ligand binding and transcriptional activation.

An expression vector (pET-LXRβ LBD) for human LXRβ LBD was constructed, in which the N-terminus of the LXRβ LBD was tagged with 10 polyhistidines. Both the wild-type and the two mutant LBDs, designated F268A and T272A, respectively, were expressed in *E. coli*. The expression levels of the protein were similar as demonstrated by Western blotting (Fig. 2A). The size of the identified protein is consistent with the predicted molecular weight of LXRβ LBD. We purified the wild-type and mutants with one step affinity chro-

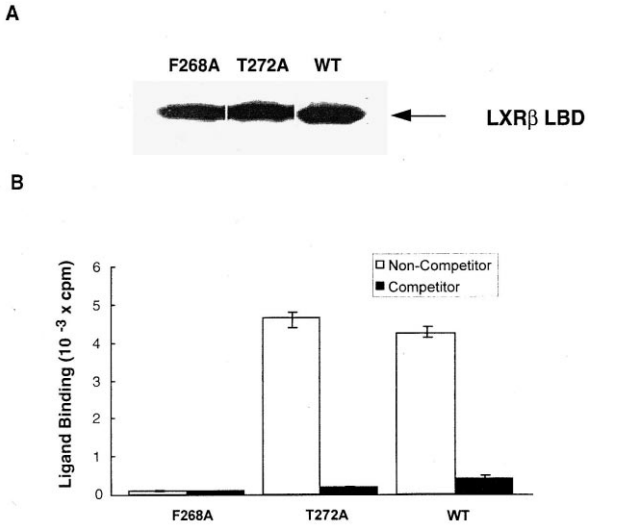


Fig. 2. Effects of point mutations on ligand binding using bacterial lysates. A: Expression of wild-type and mutant LXRβ LBDs in the presence of RXR. Same amount of lysates from *E. coli* cells expressing wild-type LXRβ LBD (lane 1), mutant F268A (lane 2) and mutant T272A (lane 3), respectively, were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to membrane. The membrane was probed with a monoclonal anti-HIS antibody and developed non-isotopically (Section 2). B: Ligand binding assay with the wild-type and mutant lysates. Same amount of lysates was used for ligand binding assay. The final [$^3$H]24(*S*)-hydroxycholesterol concentration was 200 nM. Values are mean ± S.D. ($n = 3$).

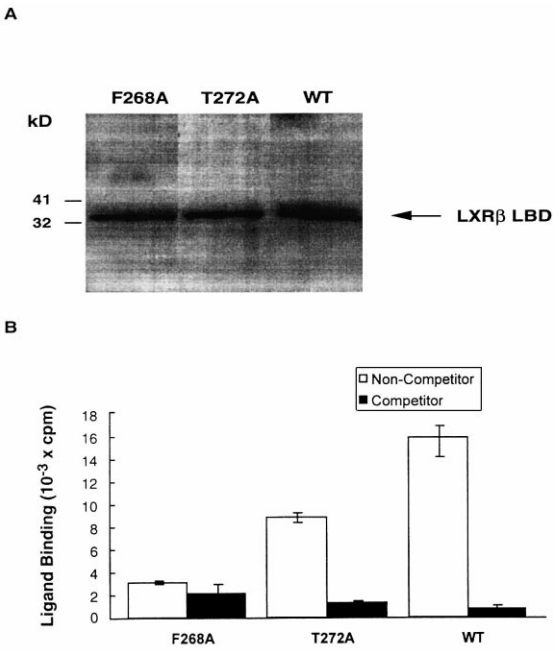


Fig. 3. Effects of point mutations on ligand binding using purified proteins. A: Purified wild-type and mutant LXRβ LBDs. Same amount of wild-type LXRβ LBD (lane 1), mutant F268A LBD (lane 2) and mutant T272A LBD (lane 3), respectively, were resolved by SDS–PAGE. B: Ligand binding assay with purified protein as described in Fig. 2. Values are mean ± S.D. ($n = 3$).

matography. The purified protein was resolved on a sodium dodecyl sulfate (SDS) gel (Fig. 3A). We found that using a washing buffer containing imidazole, the contaminating proteins and RXR can by removed from the column. This allows us to carry out a ligand binding assay with LXRβ LBD alone.

### 3.3. Ligand binding assay with the wild-type and mutant LBDs

The bacterial lysates with wild-type and mutant LBDs, respectively, were tested for ligand binding with [$^3$H]24(*S*)-hydroxycholesterol. Mutant T272A still binds at a level similar to the wild-type LBD, while F268A failed to bind [$^3$H]24(*S*)-hydroxycholesterol (Fig. 2B). These data suggest that residue F268 is critical for ligand binding.

In order to examine ligand binding in the absence of RXR, a ligand binding assay with the purified protein was carried out. Mutant F268A does not bind ligand, consistent with the data with lysates (Fig. 2B). However, mutant T272A still binds but at a lower level than the wild-type (Fig. 3B). The difference in ligand binding by T272A in the lysate and in purified form may be due to the presence of RXR. Alternatively, it could be due to changes in protein folding during or after protein purification. Nonetheless, these data suggest that residue F268 is critical for ligand binding while residue T272 is not as important. The phenylalanine residue is conserved across all the sequences in helix 3 regions of LXRs, PPARs and FXR (Fig. 1). The effect of the alanine replacement mutation at F268 might be a general effect on ligand binding instead of being specific for LXRs. Although residue T272 is unique to LXRs and FXR, it does not appear to be involved in oxysterol specific ligand binding.

### 3.4. Reporter assay with chimeric nuclear receptors

To further investigate the activities of mutants F268A and T272A, we examined the transcriptional activation by wild-type and mutant LXRβ LBDs using a reporter system [21]. A chimeric receptor was constructed with bacteria tetracycline repressor (TetR) at the N-terminus and LXRβ LBD at the C-terminus. A luciferase reporter with a TetR binding site in the promoter region (pTet-Luc) was used in the assay. The wild-type TetR-LXRβ LBD and the mutants were co-transfected with the reporter pTet-Luc in CHO cells. The transfected cells were treated with 24(*S*)-hydroxycholesterol or untreated and harvested for enzyme assay. When transfected cells were not induced with LXRβ ligand, the reporter activity was minimal (Fig. 4). In the presence of ligand, the reporter

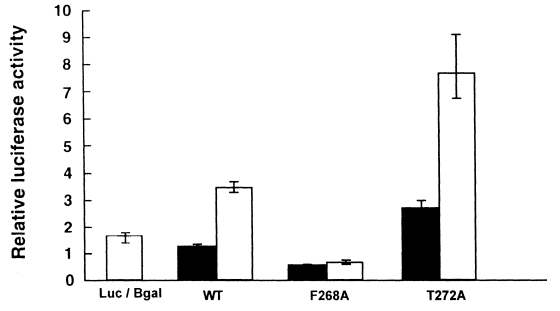


Fig. 4. Transactivation of the pTet-luc reporter gene by chimeric receptors containing LXRβ and mutant LBDs. CHO cells were transiently transfected with reporter pTet-luc, wild-type (WT) or mutant TetR-LXRβ LBDs (F268A and T272A), and a β-galactosidase expression vector (pSV-β-gal) as internal control. No chimeric receptor plasmid was included in the control group (Luc/Bgal). The transfected cells were treated with 24(*S*)-hydroxycholesterol for 24 h and harvested for luciferase and β-galactosidase assays (open bars, treated; filled bars untreated). The luciferase activity was normalized with β-galactosidase activity and expressed in arbitrary units.

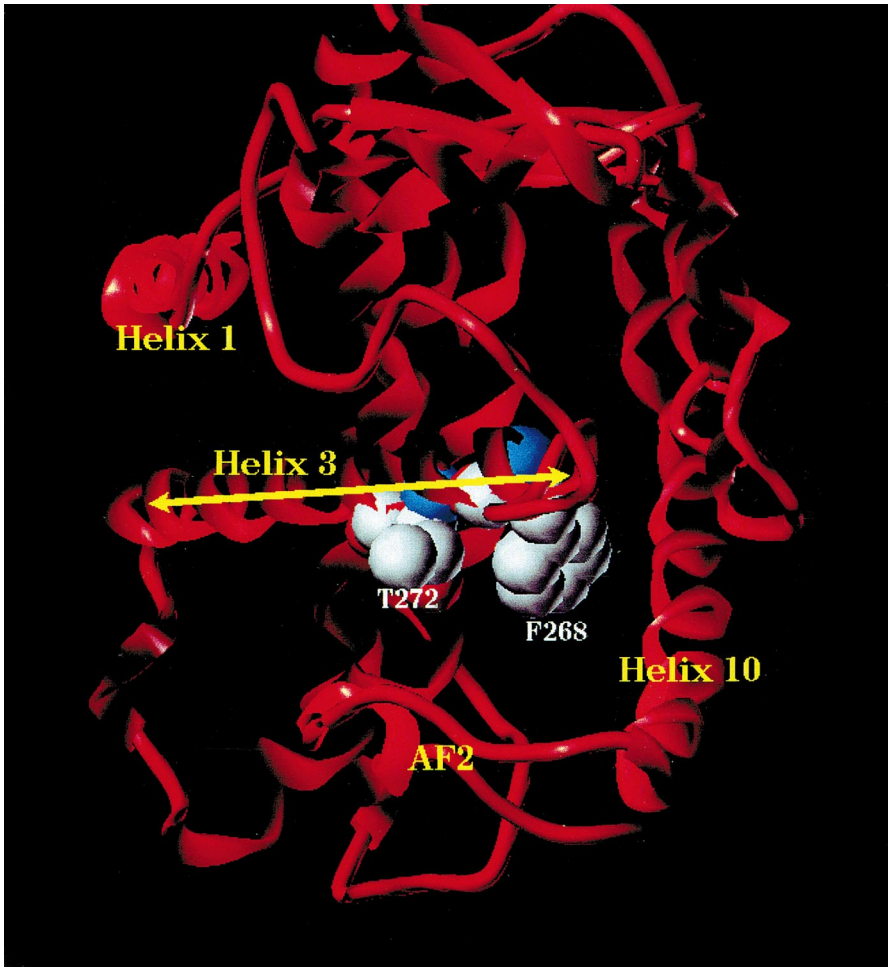


Fig. 5. A 3D model of human LXRβ. The model was built based on the sequence alignment of human LXRβ and human PPARγ and the crystal structure of human PPARγ. AF2 (helix 11), helices 1, 3 and 10 are indicated. Residues F268 and T272 in helix 3 are shown in atom to illustrate their positions relative to the ligand binding pocket.

activity was induced two-fold in cells transfected with TetR-LXRβ LBD (Fig. 4), suggesting that there is ligand dependent activation of the reporter by TetR-LXRβ LBD. In contrast, there was no transcriptional activation of the reporter by the TetR-LXRβ LBD containing the F268A mutation in the presence or absence of ligand. This data suggests that mutant F268A does not respond to the ligand and is not capable of activating gene expression, which is consistent with the ligand binding data (Figs. 2 and 3). In addition, TetR-LXRβ LBD containing mutation T272A still transactivates the reporter (Fig. 4) in the presence of ligand, consistent with the ligand binding data (Figs. 2 and 3). Therefore, mutation F268A affects both ligand binding and transcriptional activation by LXRβ while mutation T272A has little effect. It should be noted that T272A has higher basal and activation activity than the wild-type LBD. This could be due to a higher expression level of T272A. Nonetheless, the fold of activation by this mutant in the presence of ligand is similar to that by the wild-type LXRβ LBD, suggesting that mutation at T272 did not change ligand dependent activation of the reporter gene expression.

Residue F268 is a conserved amino acid across all LXRs and PPARs in the ligand entry domain. Its importance is less likely specific for LXRs but, instead, it could play a general role in ligand binding. Thus, it may not be involved in ligand selectivity. Rather, it may be involved in maintaining the structure of the ligand binding pocket.

### 3.5. The 3D model

To further explore the helix 3 region and investigate the roles of residues F268 and T272 in ligand binding by LXRβ, we built a 3D model of human LXRβ based on the published crystal structure of human PPARγ [22] and amino acid sequence alignment (Fig. 5). Both F268 and T272 reside in the helix 3 region of LXRβ. The hydrophobic side group of residue F268 faces the ligand binding pocket and might be involved in the hydrophobic interaction with oxysterols. In the meantime, F268 also faces the direction of the connecting loop between helices 10 and 11 (AF2 domain). The long side group of F268 may be required for keeping the binding pocket open by pushing the loop in the opposite direction. While the side chain of T272 is shorter it is not required for this function. Thus, removing the side chain of T272 by alanine replacement did not affect the size of the binding pocket, whereas removing the hydrophobic side group of F268 relieves the force keeping the connecting loop away from helix 3 and allows the connecting loop to move toward helix 3. As a result, the opening of the binding pocket is smaller and ligand

entry might be prevented. The role of residue F268 in ligand binding might be a combination of hydrophobic interaction with ligands and maintaining the integrity of the binding pocket structure. This is consistent with the fact that this residue is conserved in LXRs, PPARs and FXR.

### 3.6. Conclusion

Using sequence alignment and 3D modeling, we have identified residues in the helix 3 region of LXRβ LBD that might be important for ligand binding. Site directed mutagenesis studies demonstrated that residue F268 is critical for ligand binding by LXRβ while mutation at residue T272 does not affect ligand binding. These data are consistent with our findings in cell based reporter assay using chimeric receptor constructs. In contrast to the previous mutations in PPARs that change the ligand selectivity [17,24], alanine replacement at F268 completely abolished ligand binding and ligand dependent activation by LXRβ. Our data is the first study that identifies a critical residue for ligand binding and transcriptional activation by LXRβ and should provide insight into the ligand dependent activation mechanism of LXRs.

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