Structural Basis for Hydroxycholesterols as Natural Ligands of Orphan Nuclear Receptor RORγ

Lihua Jin, Dariusz Martynowski, Songyang Zheng, Taira Wada, Wen Xie, and Yong Li

Key Laboratory for Cell Biology and Tumor Cell Engineering of the Ministry of Education (L.J.), School of Life Sciences, Xiamen University, Fujian 361005, China; and Department of Pharmaceutical Sciences (L.J., D.M., S.Z., T.W., W.X., and Y.L.), Center for Pharmacogenetics, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

The retinoic acid-related orphan receptor γ (RORγ) has important roles in development and metabolic homeostasis. Although the biological functions of RORγ have been studied extensively, no ligands for RORγ have been identified, and no structure of RORγ has been reported. In this study, we showed that hydroxycholesterols promote the recruitment of coactivators by RORγ using biochemical assays. We also report the crystal structures of the RORγ ligand-binding domain bound with hydroxycholesterols. The structures reveal the binding modes of various hydroxycholesterols in the RORγ pocket, with the receptors all adopting the canonical active conformation. Mutations that disrupt the binding of hydroxycholesterols abolish the constitutive activity of RORγ. Our observations suggest an important role for the endogenous hydroxycholesterols in modulating RORγ-dependent biological processes. (***Molecular Endocrinology* 24: 923–929, 2010**)

Downloaded from https://academic.oup.com/mend/article/24/5/923/2706132 by guest on 21 August 2022

The retinoic acid-related orphan receptor γ (RORγ) is an orphan member of the nuclear receptor family that plays multiple roles in a variety of physiological processes including development, lung inflammation, circadian rhythm and lipid metabolism (1). RORγ is widely expressed in many tissues, including liver, adipose, and skeletal muscles. In mammals, RORγ is required for lymph node organogenesis and thymopoiesis (2, 3). Mice deficient of RORγ also exhibit reduced blood glucose level. Like some orphan nuclear receptors, RORγ regulates the gene transcription by binding to DNA as a monomer. RORγ contains an activation function (AF-2) located at the C terminus of its ligand-binding domain (LBD). It is believed that the precise position of the AF-2 determines the transcriptional status of a receptor. For ligand-dependent receptors, agonist binding induces the AF-2 helix in a conformation that is permissive for interactions with LXXLL motifs of coactivator proteins such as the steroid receptor coactivators (SRCs). This general mechanism is consistent with observations from several dozen crystal structures of various nuclear receptors (4–6).

RORγ is part of a nuclear receptor subfamily that includes the RORα and RORβ. Despite the appreciation of the importance of RORγ in physiology, RORγ remains an orphan because no ligands have been identified. In cell-based reporter gene assays, RORγ appears to be constitutively active because it stimulates transcription in the absence of an exogenously added ligand. However, the molecular basis that determines the RORγ activity is still elusive in the absence of a RORγ structure. In fact, RORγ is one of only a few nuclear receptors whose structures remain unsolved partly due to a lack of ligands. Nevertheless, the presence of a well-formed hydrophobic pocket predicted by modeling raises the possibility that RORγ can be regulated by physiological ligands (7, 8).

Results

The hydroxycholesterols promote RORγ/coactivator interaction

RORγ shares 48 and 46% sequence identity with RORα and RORβ in their LBDs, respectively. Because

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.
Copyright © 2010 by The Endocrine Society
doi: 10.1210/me.2009-0507 Received December 10, 2009. Accepted February 10, 2010.
First Published Online March 4, 2010

Abbreviations: AF, Activation function; 20α-HC, 20α-hydroxycholesterol; LBD, ligand-binding domain; LXR, liver X receptor; RORγ, retinoic acid-related orphan receptor γ; SRC, steroid receptor coactivator.

cholesterol has been shown to bind to RORα (8), we wanted to know whether cholesterol or its hydroxycholesterol derivatives are able to activate RORγ using the AlphaScreen biochemical assay, which is a widely used assay to detect ligand-dependent interaction between nuclear receptors and their coactivators (9, 10). The human RORγ LBD was expressed in bacteria and purified to homogeneity through nickel affinity and ion exchange chromatography. In the present study, we monitored the interaction of RORγ with a second LXXLL motif of coactivator SRC1 (SRC1-2) either in the presence or absence of cholesterol and hydroxycholesterols (Fig. 1A). RORα shows a high basal interaction with the SRC1-2 coactivator motif even in the absence of any exogenous ligands, consistent with the fact that a large fraction of the purified RORα is bound to endogenous ligands (Fig. 1B). In contrast, cholesterol and its derivatives, 20α-hydroxycholesterol (20α-HC), 22(R)-hydroxycholesterol (22R-HC), and 25-hydroxycholesterol (25-HC), strongly promoted the coactivator recruitment by RORγ. Moreover, cholesterol 20α-HC, 22R-HC, and 25-HC also strongly enhanced the interaction of RORγ with various other coactivator LXXLL motifs from the family of steroid receptor coactivators (SRC1-2 and SRC1-4), CBP, and PGC-1α but not two corepressor motifs from NcoR (NcoR-1 and NcoR-2), indicating that cholesterol and hydroxycholesterols function as RORγ agonists (Fig. 2A). Remarkably, full dose curves reveal that the potency (EC_{50}) of all three hydroxycholesterols tested are around 20–40 nM, compared with the EC_{50} of 200 nM for cholesterol (Fig. 2B), suggesting that hydroxycholesterols are highly potent RORγ ligands.

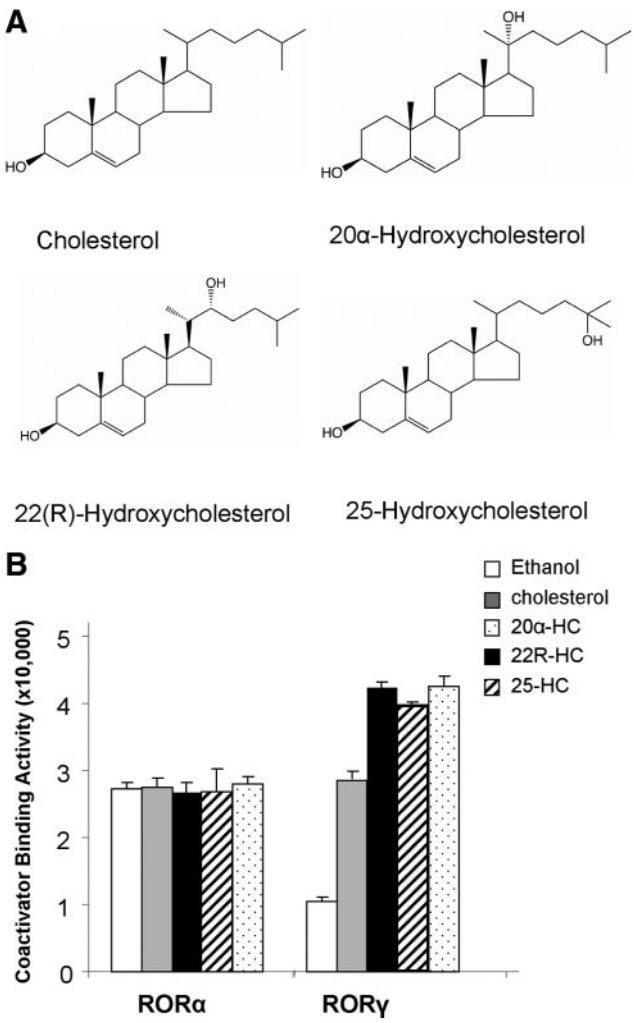

FIG. 1. The hydroxycholesterols promote RORγ/coactivator interaction. A, Chemical structures of cholesterol and various hydroxycholesterols; B, modulation of the interactions of RORs LBD with SRC1-2 coactivator LXXLL motif in response to 1 μM hydroxycholesterols by AlphaScreen assays.

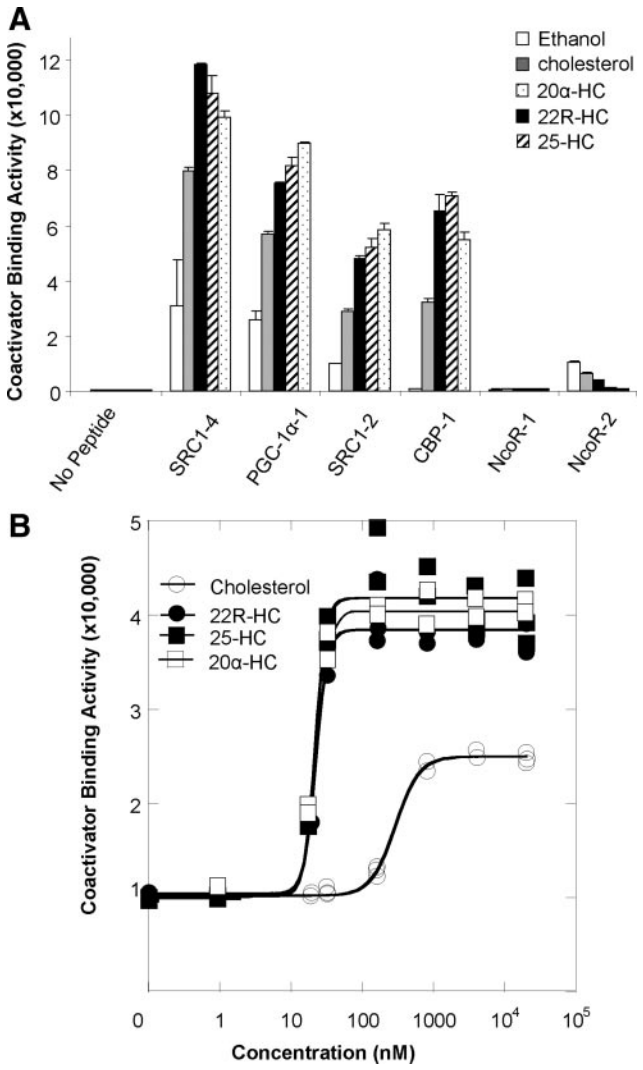

FIG. 2. The transcriptional properties of RORγ in response to cholesterol derivatives. A, Modulation of the interaction of RORγ LBD with various coactivator LXXLL motifs and corepressor motifs in response to 1 μM hydroxycholesterols as shown by AlphaScreen assays. Background reading with the RORγ LBD is fewer than 200 photon counts. The peptide sequences are listed in experimental procedures. B, Dose curve of hydroxycholesterols in promoting RORγ LBD/SRC1-2 binding by AlphaScreen assays.

Downloaded from https://academic.oup.com/mend/article/24/5/923/2706132 by guest on 21 August 2022

Structural determination of the RORγ LBD in complex with hydroxycholesterols

To determine the molecular basis for the hydroxycholesterol-regulated RORγ activity, we solved the crystal structures of RORγ LBD in complex with a SRC2 second LXXLL motif (SRC2-2) and ligands of 20α-HC, 22R-HC, and 25-HC, respectively. The data statistics and the refined structures are summarized in Table 1. Both RORγ/20α-HC and RORγ/22R-HC share the same P41212 space group, whereas the RORγ/25-HC was crystallized in P212121 space group. Figure 3A shows the overall arrangement of the RORγ complexed with 25-HC. All RORγ/20α-HC, RORγ/22R-HC, and RORγ/25-HC complexes share a highly similar LBD structure with root mean square deviation of approximately 0.5 Å for the Cα atoms. The RORγ LBD contains 12 α-helices and two short β-strands that are folded into a typical three-layer helix sandwich. Similar to other nuclear receptor LBD structures bound to coactivator LXXLL motifs, the SRC2-2 LXXLL motif adopts a two-turn α-helix that directs the three hydrophobic leucine side chains into the RORγ coactivator binding surface. The C-terminal AF-2 is positioned in the active conformation, in agreement with the agonist nature of these ligands shown by AlphaScreen assays.

The intact pocket is important for RORγ activation by hydroxycholesterols

In the RORγ structure, the ligand is completely enclosed within the bottom half of the RORγ LBD (Fig. 3A). The hydroxycholesterol is oriented with its hydroxyl tail toward helix H11 and the A ring toward helices H1 and H2. Despite the overall similarity of the structures of RORγ complexes, the electron density map calculated from the molecular replacement solution reveals clear features for the binding mode of three distinct hydroxycholesterol ligands for each complex (Fig. 3, B–D). All 20α-HC, 22R-HC, and 25-HC adopt the same well-defined position in the ligand-binding pocket, each with a unique hydroxyl group located on the specific positions of the cholesterol side chain.

To validate the roles of pocket residues in hydroxycholesterol binding and RORγ activation, we mutated several key residues that contact different portions of the bound hydroxycholesterol ligands and tested the transcriptional activity of these mutated RORγ in response to hydroxycholesterols in cell-based reporter assays. Ala327 and Phe378 are two pocket residues that contact the bulky hydrophobic cholesterol A and D rings (Fig. 4A). Two mutations were designed to either reduce the size of the RORγ pocket by changing the Ala327 to a hydrophobic but larger phenylalanine (A327F) or to shift the

TABLE 1. Statistics of data and structures

	RORγ/20α-HC	RORγ/22R-HC	RORγ/25-HC
X-ray source	APS,21ID-F	APS, 21ID-F	APS, 21ID-F
Space group	P41212	P41212	P212121
	a = b = 53.98	a = 58.50	a = 66.77
	c = 162.583	b = 58.50	b = 86.26
		c = 159.03	c = 91.95
Resolution	50–2.35 (2.4–2.35)	50–2.4 (2.46–2.4)	50–1.75 (1.79–1.75)
Total reflections	114,862	86,326	393,191
Unique reflections	10,736	11,412	52,446
Redundancy	10.7	7.6	7.5
Completeness	99.9 (99.6)	98.8 (88.0)	95.7 (61.2)
I/σ	22.7 (3.8)	17.3 (2.2)	20.6 (2.4)
R_{sym} [a]	0.122 (0.474)	0.164 (0.424)	0.096 (0.431)
Ref. statistics			
R [b]	20.0	19.4	18.6
R_{free}	29.7	28.0	25.2
RMSD bonds	0.019	0.014	0.015
RMSD angles	1.9	1.5	1.7
Nonhydrogen protein/peptide atoms	2007/104	1995/85	4104/196
Nonhydrogen ligand	29	29	58
Solvent molecules	106	168	728
Nonhydrogen protein/peptide atoms	28.4/53.4	26.5/46.9	15.9/30.8
Nonhydrogen ligand atoms	31.3	23.8	11.61
Solvent molecules	33.9	32.6	34.6

Values in *parentheses* are for highest resolution shells. RMSD, Root mean square deviation from ideal geometry of protein; APS, Advanced Photon Source.

[a] $R_{sym} = \Sigma|Iavg - Ii|/\Sigma Ii$.

[b] $R_{factor} = \Sigma|F_P - F_{Pcalc}|/\Sigma F_p$, where F_p and F_{pcalc} are observed and calculated structure factors, R_{free} was calculated from a randomly chosen 8% of reflections excluded from refinement and R_{factor} was calculated for the remaining 92% of reflections.

Downloaded from https://academic.oup.com/mend/article/24/5/923/2706132 by guest on 21 August 2022

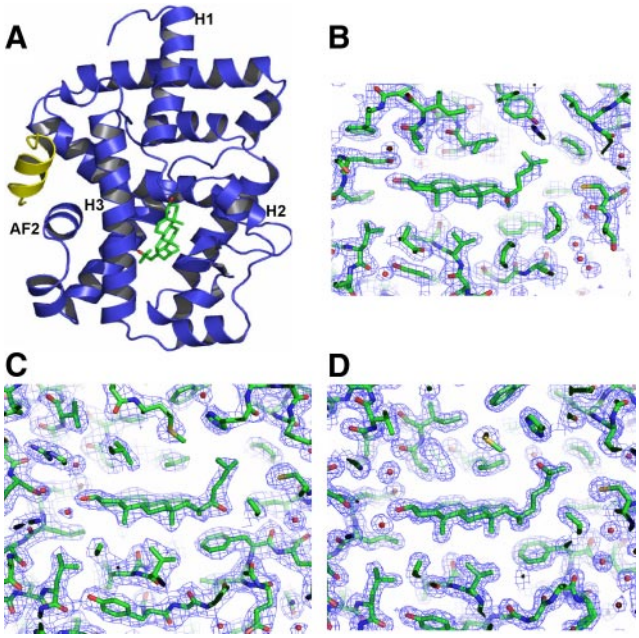

FIG. 3. The structures of the RORγ complexed with hydroxycholesterols. A, Ribbon representation of the RORγ LBD complexed with 25-HC. RORγ is in *blue*, and the SRC2-2 motif is in *yellow*. The bound 25-HC is shown in stick representation with carbon and oxygen atoms depicted in *green* and *red*, respectively. B–D, 2Fo-Fc electron density map (1.0σ) showing bound 20α-HC (B), 22R-HC (C), and 25-HC (D). All hydroxycholesterols are shown in stick representation with carbon and oxygen atoms depicted in *green* and *red*, respectively.

pocket to hydrophilic (F378Q). Accordingly, both mutations completely abolish the transcriptional activity of RORγ LBDs using a Gal4-driven promoter (Fig. 4D), highlighting the critical roles of the size and also the hydrophobic nature of the RORγ pocket in binding to ligands. To further determine the functional significance of hydroxycholesterol binding in RORγ activity, we mutated either A327 or F378 in the context of the full-length receptor RORγ and tested the ability of these mutated receptors to potentiate the activation of a RORγ reporter gene that contains the natural ROR response element (RORE) derived from the Purkinje cell protein 2 (*Pcp2*) gene (11) (Fig. 4E). Similar to Fig. 4D, the results reaffirm that the mutations that prevent hydroxycholesterol binding impair the transcriptional activity of RORγ.

In addition to 20α-HC, 22R-HC, and 25-HC, many other hydroxycholesterols, like 22S-HC and 27-HC, are also able to activate RORγ (data not shown). The observation that various hydroxycholesterols function as agonists is consistent with the size and shape of the RORγ pocket and the docking mode of all three hydroxycholesterols in the structures. Diverse cholesterol derivatives not only exist in mammalian cells but also display very high binding affinity to RORγ, which may have resulted in the high basal transcriptional activity of RORγ in cell-based reporter assays. Because RORγ is already occupied with diverse hydroxycholesterol ligands, the functional response to exogenous ligand treatment is difficult to evaluate. To study the effects of exogenously added hydroxycholesterols, we designed RORγ mutants that prevent the binding of most cholesterol derivatives but allow only specific hydroxycholesterols, 22R-HC or 25-HC, both of which are rare in cells (12). One mutation (I397N) was specifically designed to create the hydrogen bonds with the hydroxyl group at the C22 position of 22R-HC, although overall this mutation reduces the hydrophobic interactions of the RORγ pocket with the ligand (Fig. 4B). As expected, the I397N mutation resulted in nearly complete loss of RORγ transcriptional activity even in the presence of cholesterol and 25-HC because this mutant has lost the ability to bind to most endogenous cholesterol derivatives including 25-HC. In a sharp contrast, addition of exogenous 22R-HC that was predicted to form hydrogen bonds with this mutation substantially increased the RORγ transcriptional activity (Fig. 4, D and E). As a control, the mutation I397W at the same position designed to reduce the pocket size, thereby affecting all cholesterol derivatives, was not able to be activated by all the ligands tested. Similarly, L324N that was designed to selectively favor the binding of 25-HC (Fig. 4C) indeed was substantially induced by the addition of exogenous 25-HC in cell-based assays (Fig. 4, D and E). Together, our mutagenesis studies in the RORγ pocket reveal a strong correlation of hydroxycholesterol binding and RORγ activation.

Overexpression of cholesterol sulfotransferase has been shown to reduce intracellular levels of oxysterols and attenuate liver X receptor (LXR) responses (13). We therefore treated the cells with cholesterol sulfotransferase to sulfate cholesterol derivatives by transfecting pcDNA-SULT2B1b. As shown in Fig. 5, the introduction of cholesterol sulfotransferase substantially decreased RORγ transcriptional activity, suggesting the importance of the intact cholesterol derivatives for RORγ activity. Interestingly, treatment with hydroxycholesterols is able to partially restore RORγ transcriptional activity in a dose-dependent manner (Fig. 5), further supporting that hydroxycholesterols serve as RORγ ligands and regulate its transcriptional activity.

RORγ recruits coactivators via a conserved charge clamp

Upon the binding of agonists like 22R-HC and 25-HC, RORγ recruits coactivators to induce target gene transcription. The RORγ crystal structures reveal that the AF-2, together with helices H3, H4, and H5, forms a charge clamp pocket (K336 from H3 and E504 from AF-2) to interact with the SRC2 LXXLL motif (Fig. 6A),

Downloaded from https://academic.oup.com/mend/article/24/5/923/2706132 by guest on 21 August 2022

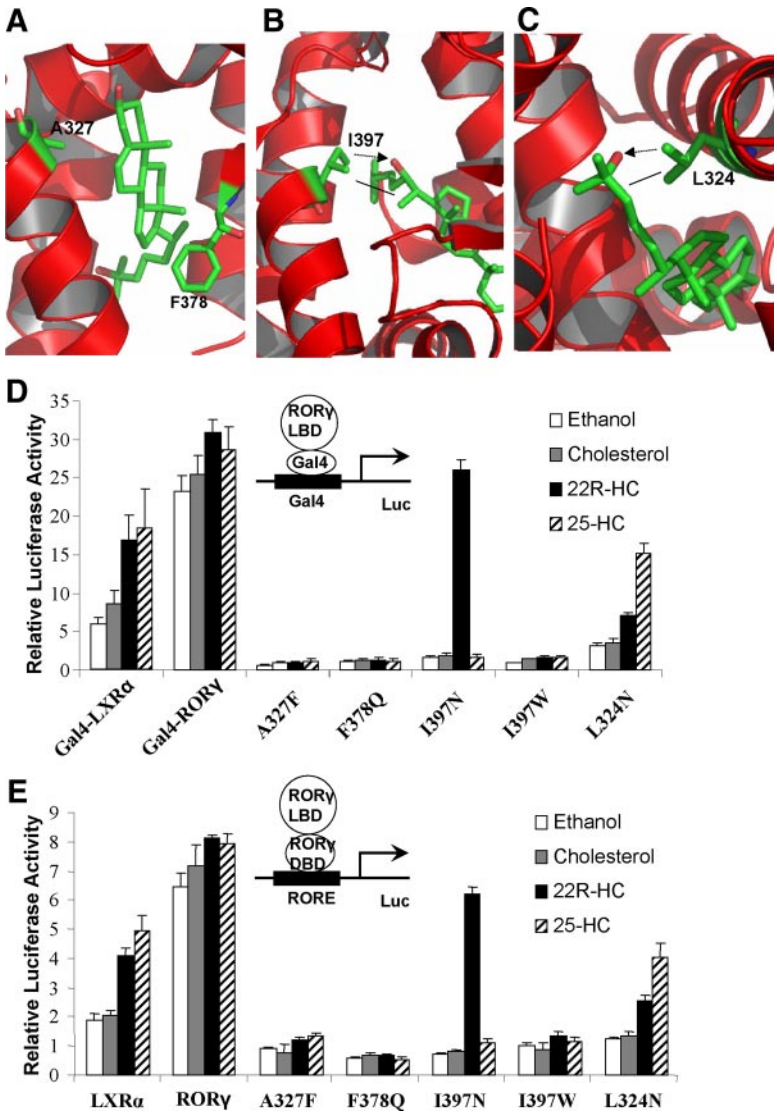

FIG. 4. The structural determinants of the interaction of RORγ LBD with ligands. A–C, The interactions of RORγ residues with specific groups on the hydroxycholesterol ligands including cholesterol rings (A) and hydroxyl groups of 22R-HC (B) and 25-HC (C). The hydrophobic interactions between RORγ and the ligands are shown with *dashed lines*. The potential hydrogen bonds, if the corresponding hydrophobic residues (Ile397 and Leu324) are mutated to Asn, are indicated by *arrows*. D and E, Effects of mutations of key RORγ residues on its transcriptional activity in cell-based reporter gene assays. The LBDs of LXRα, RORγ, and RORγ mutants were fused to Gal4 DNA-binding domain (DBD). The cells were cotransfected with pG5Luc reporter, together with the plasmids encoding Gal4-LBD fusion proteins (D). The cells were cotransfected with the *Pcp2*/RORE-Luc reporter, together with the plasmids encoding full-length LXRα, RORγ, or RORγ mutants (E), and 1 μM ligands were used in both D and E.

Discussion

The identification of ligands for orphan nuclear receptors has revealed important signaling pathways for several prominent classes of lipids including retinoids, fatty acids, and sterols (15, 16). In the current study, we have used structural and functional analysis in combination with biochemical and cell-based assays to provide strong evidence that hydroxycholesterols are high-affinity natural ligands for the orphan receptor RORγ. First, the interaction of RORγ with coactivators can be enhanced by the binding of these ligands. Second, the crystal structures of RORγ have revealed the clear binding mode of hydroxycholesterol ligands. Also the active conformation of RORγ affirms the agonist nature of these hydroxycholesterol ligands. Furthermore, the biological significance of RORγ binding to hydroxycholesterols is supported by our mutagenesis studies. Mutations that disrupt the binding of hydroxycholesterols abolish RORγ transcriptional activity, suggesting a critical role for hydroxycholesterols in activating RORγ. Moreover, mutations designed to provide hydrogen bonds with specific hydroxycholesterol ligands enhance RORγ transcriptional activity in response to these ligands accordingly. Finally, hydroxycholesterol treatment is able to partially restore RORγ transcriptional activity in cells with reduced levels of hydroxycholesterols. Taken together, we have provided coherent evidence that the transcriptional activity of RORγ is regulated by hydroxycholesterols.

Given the high concentration of various hydroxycholesterols in cells (12) and high potency of these compounds binding to RORγ, it's not surprising to see that RORγ displays high constitutive activity and does not readily respond to any exogenous added hydroxycholesterols. It is possible that various hydroxycholesterols serve as structural components for RORγ in cells, allowing the protein to achieve a desirable conformation because they are readily available. One known such example is the fatty acids binding to the HNF4 family of nuclear receptors where fatty acids are used as a structural cofactor and cannot be exchanged (17, 18). Our findings of strong correlation of hydroxycholesterol binding with RORγ activity suggest that RORγ functions as a conventional nuclear receptor by detecting appropriate ratio and balance of diverse hydroxycholesterols in the cells and adjusting

which is a conserved mode for nuclear receptors to interact with coactivators (5, 14). To test the significance of the charge clamp in RORγ activation, we mutated these two residues and tested them in cell-based reporter assays. Figure 6 shows that the mutations of either charge residue (K336E and E504K) significantly reduced RORγ activity in cell-based reporter assays when either RORγ LBD (Fig. 6B) or full-length RORγ (Fig. 6C) is used. Our results affirm the importance of the charge clamp for RORγ in recruiting coactivators to activate gene transcription.

Downloaded from https://academic.oup.com/mend/article/24/5/923/2706132 by guest on 21 August 2022

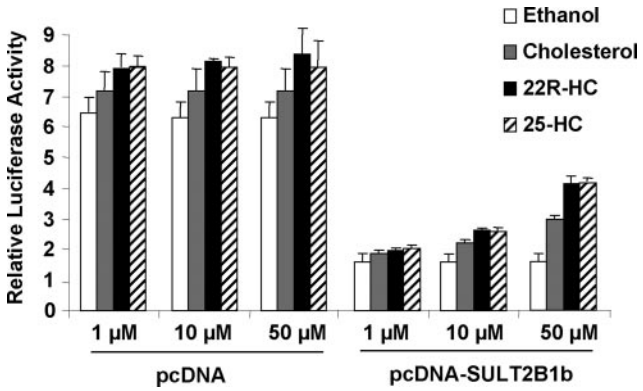

FIG. 5. Hydroxycholesterol treatment partially restores RORγ transcriptional activity in cells with reduced levels of hydroxycholesterols. Cos7 cells were transiently introduced with SULT2B1b cholesterol sulfotransferase. Then the cells were cotransfected with the *Pcp2*/RORE-Luc reporter, together with the plasmids encoding full-length RORγ. The ligands were added at the indicated concentrations.

transcription of its target genes accordingly. Interestingly, hydroxycholesterols have been shown to activate nuclear receptors LXRs that are key regulators of lipid and cholesterol homeostasis (19, 20). The identification of hydroxycholesterols as RORγ agonists has not only expanded the physiological function of cholesterol but may also provide the molecular basis by which RORγ is implicated in the regulation of lipid and steroid metabolism (11). It is interesting to note that RORs and LXRs not only share the hydroxycholesterol ligands but also have functional cross talk as we recently reported (21). Our findings will help the understanding of the relationship between the hydroxycholesterol signaling pathway and RORγ function in maintaining lipid homeostasis.

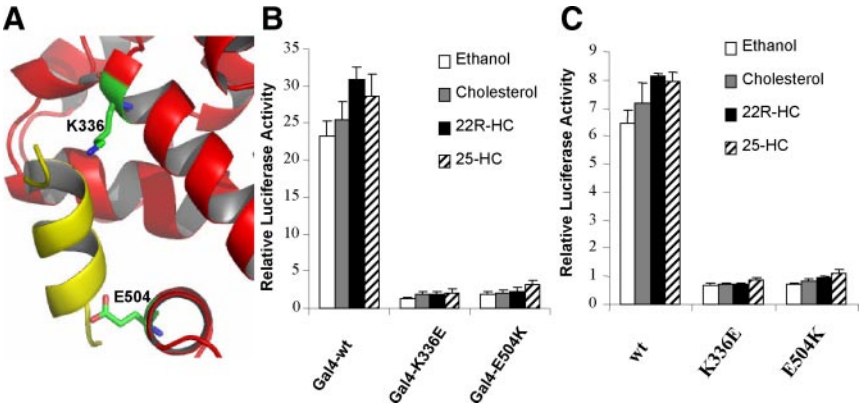

FIG. 6. Coactivators bind to RORγ via a charge clamp. A, The docking mode of SRC2-2 coactivator motif (*yellow*) on RORγ (*red*) coactivator binding site with charge clamp residues shown in stick representation. B and C, Effects of the charge clamp mutations on RORγ activity. The cells were cotransfected with pG5Luc reporter and the plasmids encoding Gal4-LBD fusion proteins (B) or the *Pcp2*/RORE-Luc reporter together with the plasmids encoding full-length RORγ or RORγ mutants (C), and 1 μM ligands were used in these assays.

Materials and Methods

Protein preparation

The human RORγ LBD (residues 262–507) was expressed as a 6xHis fusion protein from the expression vector pET24a (Novagen, Madison, WI). BL21 (DE3) cells transformed with this expression plasmid were grown in LB broth at 25 C to an OD 600 of approximately 1.0 and induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside for 16 h. Cells were harvested, resuspended, and sonicated in 200 ml extract buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, and 25 mM imadazole] per 6 liters of cells. The lysate was centrifuged at 20,000 rpm for 30 min, and the supernatant was loaded on a 5-ml $NiSO_4$-loaded HisTrap HP column (GE Healthcare, Piscataway, NJ). The column was washed with extract buffer and the protein was eluted with a gradient of 25–500 mM imidazole. The protein was further purified with a gel filtration column (GE Healthcare) with 5-fold excess of various hydroxycholesterol ligands and a 2-fold excess of the SRC2-2 peptide (KEKHKILHRLLQDSS) to the purified protein, followed by filter concentration to 15 mg/ml.

Crystallization, data collection, and structure determination

The crystals of RORγ/22R-HC complex were grown at room temperature in hanging drops containing 1.0 μl of the above protein-peptide solutions and 1.0 μl of well buffer containing 1.4 M sodium acetate, whereas the crystals of RORγ/20α-HC complex were grown in the well buffer containing 20% PEG 5000 and 150 mM ammonium sulfate. The crystals of RORγ/25-HC complex were obtained in the well buffer containing 20% PEG 3350 and 200 mM ammonium tartrate. Diffraction data were collected with a MAR225 CCD detector at the ID line of sector 21 at the Advanced Photon Source. The observed reflections were reduced, merged, and scaled with DENZO and SCALEPACK in the HKL2000 package (22). The structures were determined by molecular replacement using the crystal structure of RORα LBD as a model. Manual model building was carried out with Coot (23), followed by REFMAC refinement in the CCP4 suite (http://www.ccp4.ac.uk). The atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession codes 3KYT (RORγ/20α-HC), 3L0J (RORγ/22R-HC), and 3L0L (RORγ/25-HC).

Cofactor binding assays

The binding of the various peptide motifs to RORγ LBD and other nuclear receptors in response to ligands was determined by AlphaScreen assays using a hexahistidine detection kit from PerkinElmer (Norwalk, CT) as described (9). The experiments were conducted with approximately 20–40 nM receptor LBD and 20 nM biotinylated SRC1-2 peptide or other cofactor peptides in the presence of 5 μg/ml donor and acceptor beads in a buffer containing 50 mM MOPS, 50 mM NaF, 0.05 mM CHAPS, and 0.1 mg/ml BSA, all adjusted to a pH of 7.4.

The peptides with an N-terminal biotinylation are SRC1-2, SPSSHSSLTERHKILHR-

Downloaded from https://academic.oup.com/mend/article/24/5/923/2706132 by guest on 21 August 2022

LLQEGSP; SRC1-4, QKPTSGPQTPQAQQKSLLQQLLTE; PGC-1α-1, AEEPSLLKKLLLAPA; CBP-1, SGNLVPDAASKHKQLSELLRGGSG; NCOR-1, QVPRTHRLITLADHICQII TQDFAR; and NCOR-2, GHSFADPASNLGLEDIIRKALMGSF.

Transient transfection assay

Cos7 cells were maintained in DMEM containing 10% fetal bovine serum and were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) (24). All mutant RORγ plasmids were created using the Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The 24-well plates were plated 24 h before transfection (5×10^4 cells per well). For Gal4-driven reporter assays, the cells were transfected with 200 ng Gal4-RORγ LBD (residues 262-507), Gal4-RORγ LBD mutants, or Gal4-LXRα LBD (residues 205-447) and 200 ng pG5Luc reporter (Promega, Madison, WI). For native promoter reporter assays, the cells were cotransfected with the *Pcp2*/RORE-Luc reporter, together with the plasmids encoding full-length LXRα, RORγ, or RORγ mutants. For cholesterol sulfotransferase expression, the cells were transfected with pcDNA-SULT2B1b first to sulfate cholesterol derivatives. The cells were then transfected with indicated expression and reporter plasmids. Ligands were added 18 h after transfection. Cells were harvested 24 h later for the luciferase assays. Luciferase data were normalized to Renilla activity cotransfected as an internal control.

Acknowledgments

We thank J. S. Brunzelle for assistance in data collection at sector 21 (LS-CAT) of the Advance Photo Source and H. E. Xu for sharing the beam time. Use of the Advanced Photon Source was supported by the Office of Science of the U.S. Department of Energy.

Address all correspondence and requests for reprints to: Yong Li, Department of Pharmaceutical Sciences, Center for Pharmacogenetics, University of Pittsburgh, 633A Salk Hall, Pittsburgh, Pennsylvania 15261. E-mail: yol21@pitt.edu.

This work was supported by the National Institutes of Health Grant DK081757, an award from the American Heart Association, grants from the Science Planning Program of Fujian Province (2009J1010), and 111 Project (B06016).

Disclosure Summary: None of the authors has anything to declare regarding potential conflicts of interest.

References

1. **Jetten AM** 2009 Retinoid-related orphan receptors (RORs): critical roles in development, immunity, circadian rhythm, and cellular metabolism. Nucl Recept Signal 7:e003
2. **Kurebayashi S, Ueda E, Sakaue M, Patel DD, Medvedev A, Zhang F, Jetten AM** 2000 Retinoid-related orphan receptor γ (RORγ) is essential for lymphoid organogenesis and controls apoptosis during thymopoiesis. Proc Natl Acad Sci USA 97:10132–10137
3. **Sun Z, Unutmaz D, Zou YR, Sunshine MJ, Pierani A, Brenner-Morton S, Mebius RE, Littman DR** 2000 Requirement for RORγ in thymocyte survival and lymphoid organ development. Science 288: 2369–2373
4. **Ingraham HA, Redinbo MR** 2005 Orphan nuclear receptors adopted by crystallography. Curr Opin Struct Biol 15:708–715
5. **Li Y, Lambert MH, Xu HE** 2003 Activation of nuclear receptors: a perspective from structural genomics. Structure 11:741–746
6. **Nettles KW, Greene GL** 2005 Ligand control of coregulator recruitment to nuclear receptors. Annu Rev Physiol 67:309–333
7. **Stehlin-Gaon C, Willmann D, Zeyer D, Sanglier S, Van Dorsselaer A, Renaud JP, Moras D, Schüle R** 2003 All-*trans* retinoic acid is a ligand for the orphan nuclear receptor RORβ. Nat Struct Biol 10: 820–825
8. **Kallen JA, Schlaeppi JM, Bitsch F, Geisse S, Geiser M, Delhon I, Fournier B** 2002 X-ray structure of the hRORα LBD at 1.63 Å: structural and functional data that cholesterol or a cholesterol derivative is the natural ligand of RORα. Structure 10:1697–1707
9. **Li Y, Choi M, Cavey G, Daugherty J, Suino K, Kovach A, Bingham NC, Kliewer SA, Xu HE** 2005 Crystallographic identification and functional characterization of phospholipids as ligands for the orphan nuclear receptor steroidogenic factor-1. Mol Cell 17:491–502
10. **Motola DL, Cummins CL, Rottiers V, Sharma KK, Li T, Li Y, Suino-Powell K, Xu HE, Auchus RJ, Antebi A, Mangelsdorf DJ** 2006 Identification of ligands for DAF-12 that govern dauer formation and reproduction in *C. elegans*. Cell 124:1209–1223
11. **Kang HS, Angers M, Beak JY, Wu X, Gimble JM, Wada T, Xie W, Collins JB, Grissom SF, Jetten AM** 2007 Gene expression profiling reveals a regulatory role for RORα and RORγ in phase I and phase II metabolism. Physiol Genomics 31:281–294
12. **Schroepfer Jr GJ** 2000 Oxysterols: modulators of cholesterol metabolism and other processes. Physiol Rev 80:361–554.
13. **Chen W, Chen G, Head DL, Mangelsdorf DJ, Russell DW** 2007 Enzymatic reduction of oxysterols impairs LXR signaling in cultured cells and the livers of mice. Cell Metab 5:73–79
14. **Xu HE, Stanley TB, Montana VG, Lambert MH, Shearer BG, Cobb JE, McKee DD, Galardi CM, Plunket KD, Nolte RT, Parks DJ, Moore JT, Kliewer SA, Willson TM, Stimmel JB** 2002 Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPARα. Nature 415:813–817
15. **Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ** 2001 Nuclear receptors and lipid physiology: opening the X-files. Science 294: 1866–1870
16. **Kliewer SA, Lehmann JM, Willson TM** 1999 Orphan nuclear receptors: shifting endocrinology into reverse. Science 284:757–760
17. **Dhe-Paganon S, Duda K, Iwamoto M, Chi YI, Shoelson SE** 2002 Crystal structure of the HNF4α ligand binding domain in complex with endogenous fatty acid ligand. J Biol Chem 21:21
18. **Wisely GB, Miller AB, Davis RG, Thornquest Jr AD, Johnson R, Spitzer T, Sefler A, Shearer B, Moore JT, Miller AB, Willson TM, Williams SP** 2002 Hepatocyte nuclear factor 4 is a transcription factor that constitutively binds fatty acids. Structure 10:1225–1234
19. **Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ** 1996 An oxysterol signalling pathway mediated by the nuclear receptor LXRα. Nature 383:728–731
20. **Lehmann JM, Kliewer SA, Moore LB, Smith-Oliver TA, Oliver BB, Su JL, Sundseth SS, Winegar DA, Blanchard DE, Spencer TA, Willson TM** 1997 Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. J Biol Chem 272:3137–3140
21. **Wada T, Kang HS, Jetten AM, Xie W** 2008 The emerging role of nuclear receptor RORα and its crosstalk with LXR in xeno- and endobiotic gene regulation. Exp Biol Med (Maywood) 233:1191–1201
22. **Otwinowski Z, Minor W** 1997 Processing of x-ray diffraction data collected in oscillation mode. Methods Enzymol 276:307–326
23. **Emsley P, Cowtan K** 2004 Coot: model-building tools for molecular graphics. Acta Crystallograph D Biol Crystallograph 60:2126–2132
24. **Li Y, Suino K, Daugherty J, Xu HE** 2005 Structural and biochemical mechanisms for the specificity of hormone binding and coactivator assembly by mineralocorticoid receptor. Mol Cell 19:367–380

Downloaded from https://academic.oup.com/mend/article/24/5/923/2706132 by guest on 21 August 2022