Structural Basis for Retinoic X Receptor Repression on the Tetramer*

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Retinoic X receptor (RXR) is a master nuclear receptor in the processes of cell development and homeostasis. Unliganded RXR exists in an autorepressed tetramer, and agonists can induce RXR dimerization and coactivator recruitment for activation. However, the molecular mechanisms involving the corepressor recruitment and antagonist-mediated repression of RXR are still elusive. Here we report the crystal structure of RXR ligand-binding domain (LBD) tetramer complexed with silencing mediator for retinoid and thyroid hormone receptors (SMRT) corepressor motif. As the first structural report on the RXR dimerization and coactivator recruitment for activation, the molecular determinants for RXR repression by its corepressor, corepressor, and AF-2 motifs, whereas the AF-2 motif extends from the core structure of the LBD and makes intermolecular interactions with the coactivator-binding site of its neighboring monomer (4, 5). Such contacts thus suggest the molecular mechanism of RXR autorepression and AF-2-mediated inhibition of coactivator binding. However, interactions between RXR and the corepressors such as the nuclear receptor corepressor (N-CoR) and the silencing mediator for retinoid and thyroid hormone receptors (SMRT) were still elusive.

SMRT has been implicated in resistance to thyroid hormone, a human genetic disease characterized by an impaired physiological response to thyroid hormone (6, 7). It also links to several types of leukemia, including acute promyelocytic leukemia, acute myeloid leukemia, and pediatric β-cell acute leukemia (8). In addition, circumstantial evidence associates N-CoR and/or SMRT expression and subcellular localization with some cancers like colorectal carcinoma (9) and endometrial carcinoma (10). Recently, SMRT was reported to promote oxidative phosphorylation in adipose tissue and protect against diet-induced obesity and insulin resistance (11). Our previous study based on isothermal titration calorimetry technology showed that the SMRT corepressor motif peptide (residues...
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\( ^{2337} \text{TNMGLEAIIRKALMGK}^{2352} \) bind directly to either apo or antagonist-bound RXR LBD (5). To reveal the molecular basis for the receptor-corepressor interaction, we determine here the crystal structure of RXR LBD complexed with the SMRT corepressor motif peptide. Structural analysis indicates that four SMRT peptides bind to the tetrameric RXR LBD in a conserved binding pocket (12, 13) whereas SMRT binding unexpectedly induces a significant conformational rearrangement of the receptor compared with apoRXR LBD structure.

To elucidate further the potential molecular determinants for RXR repression by the antagonist, we also determine the crystal structure of RXR LBD-SMRT complexed with the identified antagonist rhein. Rhein is one of the major bioactive components in rhubarb (Dahuang), a famous traditional Chinese medicine derived from the rhizome of Rheum palmatum and related species. It exhibits anti-tumor, anti-inflammatory and anti-angiogenic activities (14). Rhein was also proved to be effective in the treatment of diabetic nephropathy (15, 16), and anti-angiogenic activities (14). Rhein was also proved to be effective in the treatment of diabetic nephropathy (15, 16), although no target for rhein had ever been discovered. In our current work, rhein is identified as a selective RXR antagonist, which could repress all of the tested RXR-involved homo- or heterodimeric transcription activities. The crystal structure of RXR LBD-rhein-SMRT reveals that two rhein molecules and two SMRT peptides bind to RXR LBD tetramer, which is different from the case in RXR LBD-SMRT, where four SMRT peptides are in RXR LBD tetramer. These results thus imply that rhein results in a displacement of the SMRT motif by the AF-2 motif at the corepressor-binding pocket. Further structural superposition of RXR LBDs in its apo, agonist-, and antagonist-bound states reveals that the AF-2 motif adopts different conformations catering to agonist or antagonist interaction, as well as coactivator or corepressor recruitment. Based on all these findings, we thereby propose a potential molecular model for RXR repression on the tetramer.

EXPERIMENTAL PROCEDURES

Protein Purification and Peptide Synthesis—The coding sequence of human RXR LBD (residues 221–458) was cloned to the vector pET15b, and Escherichia coli strain BL21 (DE3) was used for protein expression. The protein expression was induced with 0.5 mm isopropyl 1-thio-β-D-galactopyranoside at 25 °C for 6 h. His-tagged RXR LBD was purified with nickel-nitrilotriacetic acid resin (Qiagen), and the tag was then removed by thrombin (Novagen). The protein was further purified with Superdex 200 (Amersham Biosciences). The SMRT corepressor motif peptide was commercially synthesized with the sequence \( ^{2337} \text{TNMGLEAIIRKALMGK}^{2351} \).

Cocrystallization—All crystallization experiments were performed by hanging-drop method at 20 °C. RXR LBD was mixed with the SMRT peptide in a ratio of 1:3. Crystals grew in the condition of 100 mm sodium cacodylate, pH 6.5, 15% PEG 4000, 100 mm magnesium acetate. For the RXR LBD-rhein-SMRT complex, the ratio of protein to peptide-ligand was 1:3:5. Crystals grew in the condition of 100 mm sodium cacodylate, pH 6.5, 20% PEG 4000, 100 mm magnesium acetate.

Data Collection, Processing Structure Determination, and Refinement—Diffraction data were collected at BL17U of Shanghai Synchrotron Radiation Facility in China and integrated with HKL2000 (17). Phasing was performed with Molrep (18). Structure refinement was carried out with Refmac5 (18). Model building was manually performed with COOT (19). The statistics of the data collection and structure refinement are summarized in Table 1. Atomic coordinates and structure factors have been deposited in the Protein Data Bank under ID codes 3R29 (RXR LBD-SMRT) and 3R2A (RXR LBD-rhein-SMRT).

Luciferase Assay—Mammalian one-hybrid and transactivation experiments were performed using luciferase assays in HEK293T cells. Transient transfection was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s guideline. To evaluate the selectivity of rhein on different nuclear receptors, GAL4 DBD-RXR LBD, -PPAR γ LBD, -LXR LBD, or -FXR LBD was cotransfected with UAS-TK-Luc reporter and Renilla luciferase vector pRL-SV40. For the transactivation assays, pcDNA3.1-RXR LBD, -PPAR γ LBD, -LXR LBD, or -FXR LBD was cotransfected with pRL-SV40 plus pGL3-proRXRE-Luc, -PPRE-Luc, -LXRE-Luc, or FXRE-Luc, respectively. Five hours after transfection, cells were incubated with varied concentrations of rhein and the related positive agonist for another 24 h. RXR LBD agonist 9-cis-retinoic acid, PPAR γ agonist rosiglitazone, LXR LBD agonist T0901317, and FXR agonist chenodeoxycholic acid were used as positive agonists. Luciferase activities were then measured using the Dual Luciferase Assay System kit (Promega). Results were shown as means ± S.E., n = 3. The figures were prepared with the software Origin.

Surface Plasma Resonance Technology-based Assay—Binding affinity of rhein toward RXR LBD was determined with BIACORE 3000 instrument (BIACORE) based on our previous report (5). Briefly, RXR LBD was immobilized onto a CM5 sensor chip according to the standard primary amine-coupling procedures. Rhein was serially diluted and injected into the channels at a flow rate of 20 μl/min for 60 s followed by dissociation for 120 s.

| TABLE 1 | Data collection and refinement statistics |
|---------|-----------------------------------------|
| **Statistics** | RXR LBD-SMRT | RXR LBD-rhein-SMRT |
| **Space group** | P 4, 2, 2 | P 2, 2, 2 |
| **Cell dimensions (Å)** | a = 118.22, b = 118.22, c = 84.02 | a = 90.90, b = 90.90, c = 90.90 |
| **Resolution (Å)** | 44.7-2.9 | 42.5-3.0 |
| **Rmerge or Rmerge** | 0.092 (0.447) | 0.083 (0.542) |
| **f/i (σ)** | 49.2 (7.3) | 29.8 (4.1) |
| **Completeness (%)** | 99.5 (100) | 99.0 (100) |
| **Redundancy** | 7.5 (7.5) | 3.6 (3.8) |

*Values in parentheses are for highest resolution shell.*
RESULTS

SMRT Binding Induces a Significant Structural Rearrangement of RXR Tetramer—Our determined crystal structure of RXRαLBD-SMRT shows a tetrameric RXRαLBD conformation with one SMRT peptide bound to each receptor (Fig. 1A). The key secondary structures involving RXR activation and ligand-binding pocket are shown in Fig. 1B. Compared with the previously reported apoRXRαLBD tetramer, SMRT binding induces a significant rearrangement of the tetramer (4, 5). The two symmetry dimers in the tetramer rotate about 30° along the tetrameric axis from the position in its apo form (Fig. 1C). As a result, the atom O of Ile442 and OG1 of Thr444 in the C terminus...
of helix 10 forms two hydrogen bonds with NH1 of Arg346 in helix 7 and NH2 of Arg302 in helix 4 from its two neighboring monomers, respectively (Fig. 1D). Remarkably, the two adjacent phenylalanine residues Phe437 and Phe438 in helix 11 from each monomer of the tetramer form hydrophobic interactions in a hand-in-hand manner (Fig. 1E). Such interactions among the four helices 11 are believed to play a critical role for the tetramer stabilization. Additionally, Leu441 from each helix 11 extends itself inward to further stabilize these four helices hydrophobically (Fig. 1E). As a consequence of the tetrameric rearrangement, SMRT binding results in a much larger tetramer stabilization. Additionally, Leu441 from each helix 11 are believed to play a critical role for the tetramer stabilization. Additionally, Leu441 from each helix 11 extends itself inward to further stabilize these four helices hydrophobically (Fig. 1E). As a consequence of the tetrameric rearrangement, SMRT binding results in a much larger tetramer stabilization.

**Rhein Is Identified as a Selective RXR Antagonist**—In our previous study, danthron from the traditional Chinese medicine rhubarb was found to be a specific antagonist against RXRα (5). Rhein (Fig. 2A) is another major component of rhubarb extracts. Compared with danthron, rhein has an additional carboxyl group at the C3 site of danthron. Mammalian one-hybrid-based assays are subsequently employed to evaluate the effects of rhein on RXRα transcriptional activities. As shown in Fig. 2B, rhein inhibits the known agonist 9-cis-retinoic acid-induced RXRα transactivation by IC50 value of 0.75 μM. However, this compound exhibits no activities against all other tested nuclear receptors, including PPARγ, FXR, and LXRα (Fig. 2B). Further transactivation assays demonstrate that rhein represses not only the transcription of RXRE mediated by RXRα/RXRα homodimer, but also several kinds of RXRα-involved heterodimers, including RXRα/PPARγ, RXRα/FXR, and RXRα/LXRα heterodimers, respectively (Fig. 2C). These results thus suggest that rhein exerts its trans-repression activities on RXRα homodimer and RXRα-involved heterodimers by binding RXRα as a good selective target over other nuclear receptors.

**Rhein Binding Results in a Displacement of SMRT for AF-2 Motif**—Our previous isothermal titration calorimetry results on danthron binding to RXRαLBD showed that this antagonist bound to the receptor with a stoichiometric ratio of 1:2, and such a binding changed SMRT recruiting ratio from 1:1 to 1:2 (5). Our current crystal structure of RXRαLBD-rhein-SMRT reveals the structural basis behind these biophysical results. In this structure, two rhein molecules and two SMRT peptides are bound in RXRαLBD tetramer (Fig. 3A), different from the case in RXRαLBD-SMRT structure, where four SMRT peptides bind to RXRαLBD tetramer (Fig. 1A). Rhein binds into the...
hydrophobic ligand-binding pocket with the hydrogen bonds between OAC and OAF of rhein and O of Cys^432 in the C terminus of helix 10.

As indicated, the most significant conformational changes upon rhein binding are observed in helix 3 and the AF-2 motif, both of which are crucial for RXR activation. The N terminus of helix 3 (43VTNICQAADKQLF^55) shifts outward to adapt itself for ligand binding. Notably, rhein binding redirects the neighboring AF-2 into the SMRT binding site (Fig. 3B). The electron density map shows an unambiguous positioning of SMRT corepressor motif and the C terminus of the AF-2 motif (Fig. 3B). This observation of rhein-induced displacement of SMRT by the AF-2 motif thus confirms the competitive binding of SMRT/AF-2 to the receptor (21). Moreover, such a displacement will undoubtedly contribute to the stabilization of RXR tetramerization by an enlarged tetramer interface. Additionally, by interacting with Ile^{447} and Leu^{451} of the neighboring AF-2 motif (Fig. 3A), rhein catches this motif to stabilize the inactive tetramer further, thus totally repressing the RXR-involved transactivation (Fig. 2D).

Multiple Functions of AF-2 Motif—Interestingly, the AF-2 motif adopts different conformations for agonist, antagonist, and coregulator bindings (Fig. 4). In our previous crystal structure of bigelovin-activated RXRαLBD, the AF-2 motif overturned to seal off the ligand-binding pocket, in which Leu^{451} and Leu^{455} of the AF-2 motif played essential roles by interacting with the agonist (20). AF-2 overturning also made this motif interact directly with SRC-1 coactivator motif for RXR activation. In the autorepressed apoRXRαLBD tetramer structure, these two leucine residues (Leu^{451} and Leu^{455}) of the AF-2 motif positioned themselves into the coregulator-binding pocket of the neighboring monomer to resemble the LXXLL motif of coactivator SRC-1 for RXR autorepression (4, 5) (Fig. 1H). While in the presence of corepressor SMRT, the AF-2 motif competes with SMRT for binding to the receptor. Moreover, SMRT-induced tetrameric rearrangement makes the N terminus of AF-2 interact with SMRT, but the C terminus of the AF-2 motif becomes flexible. Upon antagonist binding, Ile^{447} and Leu^{451} of the neighboring AF-2 motif interact with the antagonist, followed by a displacement of SMRT by AF-2 binding into the coregulator-binding site (Fig. 2B). Thus, the AF-2 motif allocates different residues for agonist or antagonist interaction in the active or repressive state. It is also indicated that the AF-2 motif competes with both the coactivator and corepressor for RXR binding. As reported, removing of the AF-2 motif converted RXR to a potent transcriptional repressor (22), which might be attributed to the multiple functions of the AF-2 motif on both coregulator competition and recruitment. Therefore, it is concluded that the multifunctional AF-2 motif adopts different conformations to interact with agonist or antagonist and mediates the recruitment of both coactivator and corepressor.

DISCUSSION

Nuclear receptors comprise a superfamily of transcription factors regulating complex networks in the processes of cell
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Although helix 11 (436LFFKL441) was reported to help the tetramer stabilization of SMRT-induced RXR rearrangement, the RXR antagonist competitive binding of the AF-2 motif and SMRT corepressor absence of ligand, RXRs exist in equilibrium of the autoregulatory model of RXR repression on the tetramer (Fig. 5). In the absence of agonist, AF-2 motif sterically masks this binding site to inhibit the RXR antagonist displacement benefits the stabilization of the inactive RXR for AF-2 binding instead of helping SMRT recruitment. Such a displacement benefits the stabilization of the inactive RXR tetramer by enlarged tetrameric interface.

As shown in Fig. 1E, helix 11 plays a critical role in the tetramer stabilization of SMRT-induced RXR rearrangement. Although helix 11 (436LFFKL441) was reported to help the corepressor SMRT binding to RXR (24), both of our current structures indicated that this helix is too far from the coregulator-binding site to make direct contacts with the corepressor motif.

Another significant discovery from our current structures is about the multiple functions of the AF-2 motif. This motif occupies the coregulator-binding site in the autorepressed RXR structure and plays either a positive role in transcriptional activation by recruiting the coactivator SRC-1 or a negative role in transcriptional repression by recruiting the corepressor SMRT. Previous studies indicated that the AF-2 motif inhibited the coactivator SRC-1 binding (4), whereas the competitive binding of the AF-2 motif and corepressor SMRT to the receptor is also observed in both of our current structures. Additionally, we find that the N terminus of the AF-2 motif helps SMRT binding to the receptor by direct interactions. Structural superposition between agonist- and antagonist-bound RXR structures reveals different residues of the AF-2 motif responsible for agonist or antagonist binding (Fig. 1H). Ile447 and Leu451 are for the agonist, whereas Leu451 and Leu455 are for the agonist.

Therefore, based on all of these findings, we propose a potential model of RXR repression on the tetramer (Fig. 5). In the absence of ligand, RXRs exist in equilibrium of the autorepressed tetramers and SMRT-repressed tetramers, with the competitive binding of the AF-2 motif and SMRT corepressor motif to the coregulator-binding site. The RXR antagonist mediates the SMRT/AF-2 motif exchange with an enlarged tetramer interface. In whatever tetrameric conformations, RXRs have no transcriptional activities until the agonist-induced RXR dimerization and coactivator recruitment happen. Therefore, ligand-dependent exchange of coactivator, corepressor, and AF-2 motifs should be the fundamental regulator for RXR functioning.

REFERENCES
1. McKenna, N. J., and O’Malley, B. W. (2010) Cell 142, 822–822.e1
2. Germain, P., Chambon, P., Eichele, G., Evans, R. M., Lazar, M. A., Leid, M., De Lera, A. R., Lotan, R., Mangeldsford, D. J., and Gronemeyer, H. (2006) Pharmacol. Rev. 58, 760–772
3. Egea, P. F., Mitschler, A., Rochel, N., Ruff, M., Chambon, P., and Moras, D. (2000) EMBO J. 19, 2592–2601
4. Gampe, R. T., Jr., Montana, V. G., Lambert, M. H., Wisely, G. B., Milburn, M. V., and Xu, H. E. (2000) Genes Dev. 14, 2229–2241
5. Zhang, H., Zhou, R., Li, L., Chen, J., Chen, L., Li, C., Ding, H., Yu, L., Hu, L., Jiang, H., and Shen, X. (2011) J. Biol. Chem. 286, 1868–1875
6. Safer, J. D., Cohen, R. N., Hollenberg, A. N., and Wondsford, F. E. (1998) J. Biol. Chem. 273, 30175–30182
7. Yoh, S. M., Chatterjee, V. K., and Privalsky, M. L. (1997) Mol. Endocrinol. 11, 470–480
8. Karagianpi, P., and Wong, J. (2007) Oncogene 26, 5439–5449
9. Fernández-Majada, V., Pujadas, J., Vilardell, F., Capella, G., Mayo, M. W., Bigas, A., and Espinosa, L. (2007) Cell Cycle 6, 1748–1752
10. Uchikawa, J., Shiozawa, T., Shih, H. C., Miyamoto, T., Feng, Y. Z., Kashima, H., Oka, K., and Konishi, I. (2003) Cancer Cell 9, 2207–2213
11. Fang, S., Suh, J. M., Atkins, A. R., Hong, S. H., Leblanc, M., Nofsginer, R. R., Yu, R. T., Downes, M., and Evans, R. M. (2011) Proc. Natl. Acad. Sci. U.S.A. 108, 3412–3417
12. Xu, H. E., Stanley, T. B., Montana, V. G., Lambert, M. H., Shearer, B. G., Cobb, J. E., McKea, D. D., Galardi, C. M., Plunket, K. D., Nolte, R. T., Parks, D. J., Moore, J. T., Kliewer, S. A., Willson, T. M., and Stimmel, J. B. (2002) Nature 415, 813–817
13. Wang, L., Zuercher, W. J., Consler, T. G., Lambert, M. H., Miller, A. B., Orband-Miller, L. A., McKee, D. D., Willson, T. M., and Nolte, R. T. (2006) J. Biol. Chem. 281, 37773–37781
14. He, Z. H., He, M. F., Ma, S. C., and But, P. P. (2009) J. Ethnopharmacol. 121, 313–317
15. Jia, Z. H., Liu, Z. H., Zheng, J. M., Zeng, C. H., and Li, L. S. (2007) Exp. Clin. Endocrinol. Diabetes 115, 571–576
16. Gao, Q., Qin, W. S., Jia, Z. H., Zheng, J. M., Zeng, C. H., Li, L. S., and Liu, Z. H. (2010) Planta Med. 76, 27–33
17. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 266, 307–326
18. Collaborative Computational Project Number 4 (1994) Acta Crystallogr. D Biol. Crystallogr. 50, 760–763
19. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132
20. Zhang, H., Li, L., Chen, L., Hu, L., Jiang, H., and Shen, X. (2011) J. Mol. Biol. 407, 13–20
21. Ghosh, J. C., Yang, X., Zhang, A., Lambert, M. H., Li, H., Xu, H. E., and Chen, J. D. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 5842–5847
22. Zhang, J., Hu, X., and Lazar, M. A. (1999) Mol. Cell. Biol. 19, 6448–6457
23. Renaud, J. P., and Moras, D. (2000) Cell. Mol. Life Sci. 57, 1748–1769
24. Hu, X., Li, Y., and Lazar, M. A. (2001) Mol. Cell. Biol. 21, 1747–1758