X-ray Crystal Structures of the Estrogen-related Receptor-γ Ligand Binding Domain in Three Functional States Reveal the Molecular Basis of Small Molecule Regulation*

Received for publication, August 31, 2006 Published, JBC Papers in Press, September 21, 2006, DOI 10.1074/jbc.M608410200

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The nuclear receptor (NR)² superfamily comprises 48 ligand-regulated transcription factors in the human genome.

X-ray crystal structures of the ligand binding domain (LBD) of the estrogen-related receptor-γ (ERRγ) were determined that describe this receptor in three distinct states: unliganded, inverse agonist bound, and agonist bound. Two structures were solved for the unliganded state, the ERRγ LBD alone, and in complex with a coregulator peptide representing a portion of receptor interacting protein 140 (RIP140). No significant differences were seen between these structures that both exhibited the conformation of ERRγ seen in studies with other coactivators. Two structures were obtained describing the inverse agonist-bound state, the ERRγ LBD with 4-hydroxytamoxifen (4-OHT), and the ERRγ LBD with 4-OHT and a peptide representing a portion of the silencing mediator of retinoid and thyroid hormone action protein (SMRT). The 4-OHT structure was similar to other reported inverse agonist bound structures, showing reorientation of phenylalanine 435 and a displacement of the AF-2 helix relative to the unliganded structures with little other rearrangement occurring. No significant changes to the LBD appear to be induced by peptide binding with the addition of the SMRT peptide to the ERRγ plus 4-OHT complex. The observed agonist-bound state contains the ERRγ LBD, a ligand (GSK4716), and the RIP140 peptide and reveals an unexpected rearrangement of the phenol-binding residues. Thermal stability studies show that agonist binding leads to global stabilization of the ligand binding domain. In contrast to the conventional mechanism of nuclear receptor ligand activation, activation of ERRγ by GSK4716 does not appear to involve a major rearrangement or significant stabilization of the C-terminal helix.

Amino acid sequence alignment identifies several conserved regions within the NRs: a central, highly conserved DNA binding domain that allows for the sequence-specific recognition of DNA in the promoter region of the genes; a C-terminal ligand binding domain (LBD) that is able to bind small, lipophilic molecules and influence the interaction with cofactors involved in transcriptional regulation; and a poorly conserved N-terminal domain. X-ray crystallography has revealed that the canonical NR LBD is composed of 10–13 α-helices arranged in a three-layered sandwich, the interior of which varies in volume and functional character to define the molecular basis of ligand specificity.

The estrogen-related receptors (ERRα, ERRβ, and ERRγ, or NR3B1, NR3B2, and NR3B3, respectively) define a subfamily of three orphan NRs most closely related to the classic estrogen receptors (ERα and ERβ). Because of high sequence conservation within the DNA binding domains of the two subfamilies, the classic ERs and orphan ERRs are able to recognize common DNA binding sites (also known as response elements) proximal to target genes (1). For example, the orphan ERRs have demonstrated control over classic ER target genes in breast (2) and bone (3). Although the ERs and ERRs show a degree of sequence identity across their LBDs, a significant difference in ligand binding is observed: the ERs bind with high affinity to phenolic steroids such as estradiol, whereas the ERRs are not activated by classic estrogens even at concentrations several orders of magnitude above physiological levels.

ERRγ, like ERRα and ERRβ, shows transcriptional activation of target genes in the absence of an added ligand and has been designated a constitutively active orphan NR. An x-ray crystal structure of the unliganded (apo) receptor shows the LBD in an active conformation with the C-terminal AF-2 helix oriented to allow for coactivator binding (4). The ligand binding pocket is extremely small, measuring 280 Å³ in volume compared with 370 Å³ available in the classic ERα pocket (5). An endogenous small molecule ligand has yet to be identified for ERRγ (6). Instead, the fasting-induced coactivator protein PGC-1α has been shown to activate the receptor (7–9). Taken together, these observations have led to speculation that the endogenous activity of the receptor may be controlled by coactivator concentration rather than a small molecule hormone.

Although a natural ligand remains to be found, several synthetic ligands have been identified that bind to and influence the functional activity of ERRγ. The classic high affinity ER ligands (see Fig. 1) diethylstilbestrol (1) and 4-hydroxytamoxifen (2, 4-OHT) profile as ERRγ inverse agonists (10). X-ray
crystal structures of 1 and 2 bound to ERRγ have been reported, and both structures are consistent with the observed inverse agonism (11). Due to the small volume of the ligand pocket, the receptor undergoes a large conformational change upon binding 1 or 2 that displaces the AF-2 helix resulting in a loss of coactivator binding. The global conformational change is similar to that observed when 4-OHT binds to ERα, where 4-OHT also inhibits receptor activity; however, in the more restricted ERRγ ligand binding space, the small ERα agonist diethylstilbestrol requires rearrangement within the ERRγ ligand binding pocket and profiles as an inverse agonist.

We recently reported the discovery of the first small molecule ERR agonist, GSK4716 (shown in Fig. 1 as compound 3) through a combination of diversity screening and structure-guided array synthesis (12). The acyl hydrazone is a micromolar ERR agonist that is able to activate the receptor to the same degree as the protein ligand PGC-1α. Radioligand competition binding assays show that GSK4716 is more than 50-fold selective for binding to ERRγ compared with the classic ERs (12). Given that unliganded ERRγ resides in a transcriptionally active conformation, we sought an understanding of the molecular basis of ERRγ activation by GSK4716. Herein we report x-ray crystal structures involving ERRγ in three distinct states: unliganded, inverse agonist-bound, and agonist-bound. These structures and associated studies reveal the origin of the binding selectivity and functional activity of GSK4716.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Human ERRγ LBD (229 – 458) was cloned into pRSET vector as a His6-tagged protein and expressed in *Escherichia coli* BL21(DE3) cells. Following cell growth in Circle Grow (Bio 101 system) media containing 100 mg/ml carbenicillin, cells were pelleted and stored at −80 °C. Cell pellets were lysed by resuspension and sonication in 25 mM Tris, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, and 5 mM diethiothreitol. The lysate was clarified by centrifugation, and imidazole was added to the supernatant to a final concentration of 50 mM. The protein solution was filtered and loaded onto a 50-ml nickel-nitrilotriacetic acid (Qiagen) column. Bound protein was eluted with a 50 mM to 500 mM imidazole linear gradient in 25 mM Tris, pH 8.0, 150 mM NaCl over 13 column volumes, and the ERRγ-containing fractions were pooled according to SDS-PAGE analysis. The pooled material was diluted 5-fold with 25 mM Tris, pH 8.0, 5% propanediol, 0.5 mM EDTA, and 5 mM diethiothreitol. Diluted protein was loaded onto a 50-ml Poros HQ 50 column (PerSeptive Biosystems) and eluted with a 25 to 250 mM NaCl gradient over 18 column volumes. Pooled material was further purified using an S-75 column with an elution buffer containing 5% propanediol, 25 mM Tris, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, and 5 mM diethiothreitol. Peak fractions were collected and concentrated to 8 mg/ml. The final material was aliquoted, flash frozen in liquid N2, and stored at −80 °C in the S-75 column buffer.

**CD Spectroscopy**—Purified ERRγ LBD was buffer exchanged into phosphate-buffered saline, pH 8.0, using G-25 gel-filtration chromatography for use in CD experiments. Ligands were dissolved in ethanol, and synthetic peptides were dissolved in phosphate-buffered saline, pH 8.0, before being dispensed into the protein solution to achieve referenced concentrations. Ellipticity was measured for samples in rectangular quartz cuvettes with 1.0-mm path lengths using an Aviv 62DS spectropolarimeter equipped with a four-position Peltier temperature-controlled sample turret. For thermal sensitivity experiments, the temperature was varied and measured in the sample compartment at approximately one-degree increments, generally from 25 °C to 70 °C, and ellipticity at 222 nm (bandwidth = 1 nm) was measured. Samples were equilibrated for 30 s at each temperature before data were collected at 0.1-s intervals and averaged over 2–5 s. All thermal transitions observed for ERRγ LBD led to irreversible precipitation of the protein, so the loss of ellipticity with increasing temperature was monitoring a combination of secondary structural unfolding, aggregation, and precipitation from solution. Nonetheless, certain compounds and peptides did have an effect on the observed thermal transition of ERRγ LBD indicating that this observation of thermal sensitivity was reflective of complex formation, and thereby related to the stabilization of the protein by ligand or peptide binding. Thermal transitions were analyzed using nonlinear regression to the Boltzmann equation,

\[
\Theta^T = \Theta^{\text{min}} + \frac{\Theta^{\text{max}} - \Theta^{\text{min}}}{1 + e^{(T - T_{1/2})/\Delta x}}
\]  

(Eq. 1)

where \(\Theta^T\) is the ellipticity at 222 nm measured at each temperature, \(T\), \(\Theta^{\text{min}}\) is the minimal ellipticity, \(\Theta^{\text{max}}\) is the maximal ellipticity, \(T_{1/2}\) is the midpoint of the thermal transition, and \(\Delta x\) is the slope of the transition.

**Crystallization**—Purified ERRγ protein in S-75 purification buffer was complexed with a 3-fold molar excess of compounds 4-OHT or GSK4716 (50 mM stock in Me2SO) for 1.5 h at room temperature (with or without an equimolar amount of 1.5 mg/ml of peptide). The resulting protein or protein complexes were filtered through a 0.2-μm filter prior to crystallization to remove any precipitated material. Crystallization trials using commercial screens for each protein complex were carried out using a hanging-drop vapor diffusion method in which drops containing 2 μl of protein solution and 2 μl of mother liquor
solution were equilibrated above a 1-ml mother liquor reservoir. Different temperatures and conditions were required to optimize the crystal growth for each protein complexes. The optimal crystallization condition found for unliganded ERR\textgamma protein was at 22°C with a well buffer containing 100 mM Tris, pH 8.0, 15 mM CaCl\textsubscript{2}, 30% (w/v) PEG600, and 2% (w/v) PEG35K. The ERR\textgamma complex containing a peptide representing residues 366–390 of receptor interacting protein 140 (RIP140) were grown at 22 °C with a well buffer containing 0.2M LiOAc and 7% (w/v) PEG3350 and 0.2M sodium formate.

**RESULTS**

**X-ray Crystallography**—Crystal structures of the ERR\textgamma LBD were determined in three functional states: unliganded (both in the presence and absence of an LXXLL motif cofactor peptide from RIP140), bound to the agonist GSK4716 (with the same RIP140 peptide), and bound to inverse agonist 4-OHT (both in the presence and absence of a SMRT peptide).\textsuperscript{3} A summary of the crystallographic data and refinement statistics appears in Table 1.

| Data collection and refinement statistics | ERR\textgamma (unliganded) | ERR\textgamma + Rip140 | ERR\textgamma + GSK4716 + Rip140 | ERR\textgamma + 4-OHT | ERR\textgamma + 4-OHT + SMRT |
|------------------------------------------|----------------------------|------------------------|-------------------------|------------------|----------------------|
| Space group                              | P\textsubscript{2}1,2,2    | P\textsubscript{4}1,2   | R3, hexagonal setting   | P\textsubscript{4}1,2 | C2                   |
| Cell (Å)                                 | 60.39                      | 64.32                  | 203.81                  | 64.01            | 200.22               |
| Beta value (°)                           | 80.37                      | 64.32                  | 203.81                  | 64.01            | 117.17               |
| Molecules/asymmetric unit                | 211.22                     | 139.13                 | 53.46                   | 138.23           | 90.47                |
| Data resolution (Å)                      | 4.00                       | 1.95                   | 2.60                    | 1.70             | 2.85                 |
| Average redundancy                       | 13.57 (1.39)\textsuperscript{a} | 43.26 (5.95)          | 39.53 (2.51)            | 30.70 (3.94)     | 12.53 (2.21)         |
| Rmerge (%)                               | 7.43 (57.0)                | 5.7 (37.9)             | 6.4 (40.3)              | 7.0 (35.1)       | 9.3 (29.8)           |
| Completeness (%)                         | 90.5 (61.1)                | 99.8 (99.4)            | 98.8 (84.9)             | 99.0 (99.7)      | 90.2 (39.9)          |
| R1 (%)                                   | 3.2 (2.2)                  | 13.1 (9.2)             | 9.5 (3.2)               | 12.9 (8.5)       | 1.8 (1.4)            |
| Unique reflections                       | 31,162                     | 22,078                 | 25,066                  | 32,333           | 80,155               |
| R-factor (%)                             | 20.7                       | 18.2                   | 23.2                    | 18.0             | 21.8                 |
| Free R-factor (%)                        | 26.3                       | 23.1                   | 25.7                    | 19.5             | 27.8                 |
| Protein atoms                            | 7,172                      | 19,94                  | 3760                    | 1951             | 10620                |
| Solvent atoms                            | 253                        | 282                    | 25                      | 330              | 61                   |
| Ligand atoms                             | 0                          | 0                      | 21                      | 29               | 174                  |
| Mean B-factor (Å\textsuperscript{2})     | 39.2                       | 29.6                   | 44.6                    | 21.8             | 32.7                 |
| Ramachandran statistics                  |                            |                        |                         |                  |                      |
| Core region (%)                          | 94.1                       | 94.9                   | 92.2                    | 94.6             | 93.5                 |
| Allowed region (%)                       | 5.9                        | 5.1                    | 7.6                     | 5.4              | 6.3                  |
| Generous region (%)                      | 0.0                        | 0.0                    | 0.2                     | 0.0              | 0.1                  |
| r.m.s.d. from ideal values               |                            |                        |                         |                  |                      |
| Bonded B-factors (Å\textsuperscript{2})  | 0.68                       | 1.40                   | 0.83                    | 1.27             | 0.54                 |
| Bond lengths (Å)                         | 0.010                      | 0.011                  | 0.011                   | 0.011            | 0.010                |
| Bond angles (°)                          | 1.16                       | 1.13                   | 1.22                    | 1.35             | 1.21                 |
| Torsion angles (°)                       | 4.35                       | 4.42                   | 5.58                    | 4.52             | 5.19                 |

\textsuperscript{a} Numbers in parentheses represent values for the high resolution shell.

\textsuperscript{3} K. P. Madaus, E. T. Grygielko, S.-J. Deng, A. C. Sulpizio, T. B. Stanley, C. Wu, S. A. Short, S. K. Thompson, E. L. Stewart, N. J. Laping, S. P. Williams, and J. D. Bray, manuscript in preparation.
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Unliganded Structures—In the absence of a cofactor peptide, unliganded ERRγ crystallized as a dimer with two dimers in each asymmetric unit. ERRγ LBD adopts the canonical three-layered α-helical sandwich observed in other NR structures. In complex with a fragment of NR cofactor RIP140, apo-ERRγ crystallizes with one molecule per asymmetric unit, however, the same dimer is formed by a crystallographic 2-fold axis. The crystal structures of unliganded ERRγ LBD in the presence or absence of the RIP140 fragment are similar (r.m.s.d. of Ca positions = 0.36 Å), and in both cases no density was observed in the pocket indicating that no small molecules (either carried along during the protein purification or from the crystallization buffer) were specifically bound. The RIP140 peptide is highly ordered between residues 378 and 386, which spans the region containing the NR-box LXXLL motif. The coactivator interacts via a charge clamp comprising Lys-284 on helix 3 and Glu-452 on the AF-2 helix. The ERRγ-RIP140 complex is similar to the previously reported apo-ERRγ-SRC1 complex (4). An overlay of the Co traces results in an r.m.s.d. of 0.38 Å for the Cα atoms in the ligand binding domains, and 0.39 Å for the Cα atoms in the two LXXLL peptides.

Although most of the residues lining the ligand binding pocket are identical between the classic ERα and orphan ERRγ, a few changes in corresponding residues lead to the decrease in volume available for ligand binding. The most significant difference is ERRγ Phe-435, which corresponds to ERα Leu-525 and defines one side of the ERRγ pocket, preventing ERRγ from binding to common steroidal estrogens. More subtle changes that affect the available volume for ligand binding and ligand binding pocket topology include Ala-431 and Phe-450, which correspond to ERα Gly-521 and Leu-540, respectively. The volume of this apo-ERRγ ligand binding pocket, which is the pocket on the lower left in Fig. 2, was calculated with the GRASP/MVP procedure as 280 Å³. For comparison, the ERα ligand binding pocket has a volume of 480 Å³, as calculated with the same procedure. The GRASP/MVP procedure uses a fine grid and tends to return slightly higher calculated volumes than some other procedures, which may account for the differences in pocket volumes to those previously reported for these proteins. It is conceivable that small, phenol-containing molecules could bind to ERRγ, because the key phenol binding residues are conserved between the receptors (ERRγ Glu-275 and Arg-316 and ERα Glu-353 and Arg-394). Aside from the ligand binding pocket, there is also another pocket, toward the upper right in Fig. 2, with a volume of 390 Å³. This pocket is separated from the ligand binding pocket by Glu-275 and Arg-316.

Inverse Agonist Complexes—The complex of ERRγ with the inverse agonist 4-OHT was crystallized with 1 molecule in the asymmetric unit but still forms a homodimer across a 2-fold axis in the crystal. Electron density for the 4-OH ligand is shown in Fig. 3A. The receptor adopts the canonical NR fold, and there are no significant conformational changes in helices 1–10 when compared with the apo crystal structures. However, the inverse agonist ligand binding significantly affects the end of helix 10 and the AF-2 helix. Phe-435 adopts a conformation different from that in the apo-ERRγ structure. The rotation of Phe-435 leads to a steric clash with Leu-454 and Phe-450 on the AF-2 helix. As a result, the AF-2 helix is displaced from its position capping the ligand binding pocket and appears to be partially disordered. The major AF-2 helix conformation visible in the electron den-

![FIGURE 2. Structure of the unliganded ERRγ ligand binding domain.](image)

![FIGURE 3. Ligand density for inverse agonist bound structures.](image)
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density is closely related to that previously observed in the ERα-4-OHT structure, but it appears to exist in that conformation at no more than a 60% occupancy level. The displacement of the AF-2 helix would block the binding of any NR-box coactivator peptides. A previously reported structure of ERRγ-4-OHT involves similar placement of AF-2 helix. As in the ERα-4-OHT crystal structure, Glu-275 and Arg-316 (ERα Glu-353 and Arg-394) interact with the 4-hydroxy group of the ligand, confirming the functional conservation of the phenol binding site between the classic ERs and orphan ERRs.

The complex between ERRγ, 4-OHT, and a SMRT peptide consists of six molecules in the asymmetric unit which effectively forms three homodimers. All six of the LBDs contain the 4-OHT ligand, which interacts as observed in the ERRγ-4-OHT complex. The electron density for one of the ligand molecules is shown in Fig. 3B. The AF-2 helix is significantly displaced from its position capping the binding pocket, with one molecule in each dimer having the AF-2 helix completely disordered. The other molecule in each dimer has a well ordered AF-2 helix, which interacts across a 2-fold axis with a molecule in a neighboring homodimer that has a disordered AF-2 helix. This interaction of the two homodimers in this fashion effectively makes a tetramer tied together by the interactions of the two AF-2 helices (Fig. 4). The 2-fold axis that relates these molecules is noncrystallographic for two of the homodimers in the asymmetric unit and crystallographic for the third homodimer, which forms a tetramer with a symmetry copy of itself in a neighboring asymmetric unit. One LBD from each of the homodimers binds the ordered region of a SMRT peptide (residues 1319 and 1330). The peptide interacts with the LBD in a manner similar to that seen in the peroxisome proliferator-activated receptor α-SMRT complex with the hydrophobic face of the amphipathic helix lying across the LBD near the ligand in a manner that would be prohibited by the AF-2 helix if it were in the agonist conformation (23). Lys-284 from ERRγ provides a single “charge clamp” to the end of the SMRT helix via the backbone carbonyl of Leu-1328. The AF-2 helix interacts with the neighboring LBDs in much the same way we see the SMRT peptide in this complex interacting with residues 446–455 having an r.m.s.d. of 2.0 Å between Cα atoms relative to the residues 1320–1329 of the SMRT peptide. The larger hydrophobic side chains found on the AF-2 helix appear to be responsible for the shift in position relative to the SMRT peptide; however, this does not prevent Lys-284 from binding to the carbonyl of Met-453 on the AF-2 helix.

Agonist Complex—The structure of a ternary complex of ERRγ-RIP140 with the small molecule agonist GSK4716 reveals a homodimer of ERRγ, with the AF-2 helix in both molecules adopting the agonist conformation. The agonist ligand is visible in the pocket of one of the two molecules in the homodimer (Fig. 5). The global receptor conformation and coactivator binding are similar to the unliganded ERRγ structure. Notably, Phe-435 and Phe-450 do not rotate from their apo conformation to accommodate the binding of the agonist ligand. Instead, a small rearrangement of the receptor (overall Cα r.m.s.d. of 0.91 Å to the RIP140 structure) occurs primarily from the N terminus of helix 1 to the beginning of helix 3. There is marked movement of three residues, in particular Pro-246, Glu-247, and Lys-248, with Pro-246 showing the greatest movement (~7.5 Å) relative to the unliganded conformation. As a result of this reorganization, there is an observable difference in the structure between helices 1 and 3 when comparing the unliganded and agonist-bound ERRγ structures (Fig. 6). The phenol-binding residues Glu-275 and Arg-316 are rotated to allow access to an additional pocket that was previously shielded from the

FIGURE 4. ERRγ-4-OHT-SMRT complex. The tetrameric assembly containing four of the six molecules in the asymmetric unit is shown. A ribbon representation of the ERRγ monomers is shown with each monomer shown with a distinct color: yellow, molecule A; magenta, molecule B; blue, molecule C; and green, molecule D. The ligand is depicted as a stick figure with orange carbon atoms. Residues 319–330 of the SMRT peptide are depicted as cyan ribbons with peptide chain G binding ERRγ molecule D. The ligand is depicted as a green ribbon. The AF-2 helix is significantly displaced from its position capping the binding pocket, with one molecule in each dimer having the AF-2 helix completely disordered. The other molecule in each dimer has a well ordered AF-2 helix, which interacts across a 2-fold axis with a molecule in a neighboring homodimer that has a disordered AF-2 helix. This interaction of the two homodimers in this fashion effectively makes a tetramer tied together by the interactions of the two AF-2 helices (Fig. 4). The 2-fold axis that relates these molecules is noncrystallographic for two of the homodimers in the asymmetric unit and crystallographic for the third homodimer, which forms a tetramer with a symmetry copy of itself in a neighboring asymmetric unit. One LBD from each of the homodimers binds the ordered region of a SMRT peptide (residues 1319 and 1330). The peptide interacts with the LBD in a manner similar to that seen in the peroxisome proliferator-activated receptor α-SMRT complex with the hydrophobic face of the amphipathic helix lying across the LBD near the ligand in a manner that would be prohibited by the AF-2 helix if it were in the agonist conformation (23). Lys-284 from ERRγ provides a single “charge clamp” to the end of the SMRT helix via the backbone carbonyl of Leu-1328. The AF-2 helix interacts with the neighboring LBDs in much the same way we see the SMRT peptide in this complex interacting with residues 446–455 having an r.m.s.d. of 2.0 Å between Cα atoms relative to the residues 1320–1329 of the SMRT peptide. The larger hydrophobic side chains found on the AF-2 helix appear to be responsible for the shift in position relative to the SMRT peptide; however, this does not prevent Lys-284 from binding to the carbonyl of Met-453 on the AF-2 helix.

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FIGURE 5. ERRγ-GSK4716-RIP140 Complex. A, the ligand binding pocket of the first LBD of the ERRγ-GSK4716-RIP140 complex is shown. The GSK4716 ligand was clearly visible from the original molecular replacement density in this LBD. The refined ligand model is shown along with a 2Fo − Fc electron density map surrounding the ligand calculated at 2.60 Å and contoured at a level of 2.0 σ. The salt bridge normally present in the apo structure between Arg-316 and Glu-275 is disrupted, B, the ligand binding pocket of the second LBD in the asymmetric unit is shown. A 2Fo − Fc electron density map contoured at 1.0 σ is shown in blue. Residues from a symmetrically related molecule in the crystal are shown in green. An Fc − Fo electron density map calculated at 2.6 Å is also shown with +3 σ contours shown in green and −3 σ contours shown in red. There is only a hint of ligand occupancy in this LBD at very low contours, so we have modeled this LBD without ligand.
classic estrogen binding site in a location remote from the AF-2 helix, which had a volume of 390 Å³. The movement of phenolic binding residues and those centered around Pro-246 substantially change the shape of the original remote second pocket and does not make contact with Glu-275 and Arg-316. GSK4716 interacts with Asp-328 near the surface of the receptor, with selected side chains depicted using green carbon, red oxygen, and blue nitrogen atoms. The most notable movement in the main chain upon binding of compound occurs in the region just below helix 1 involving residues 246–248, although some movement of all residues N-terminal to helix 3 was observed. The volume available to the ligand in the pocket of the ERRγ-GSK4716-RIP140 complex is depicted with a semitransparent surface. This induced pocket, which has a volume of 610 Å³, resulted from a merging of the two pockets shown for the apo receptor in Fig. 2, which occurred when the salt bridge between Asp-275 and Arg-316 was disrupted. The portion of the combined pocket contributed by the apo pocket 1 remained fairly static, whereas there was significant rearrangement of the residues that lined the apo pocket 2 changing its shape.

**TABLE 2**

| Complex          | $T_{1/2}$ | S.D.$^a$ | n$^b$ |
|------------------|----------|---------|------|
| ERRγ             | 48.3     | 0.1     | 7    |
| ERRγ-GW716       | 49.2     | 0.1     | 3    |
| ERRγ-RIP140      | 51.3     | 0.5     | 6    |
| ERRγ-GW716-RIP140| 52.5     | 0.4     | 6    |
| ERRγ-4-OHT       | 55.0     | 0.1     | 4    |
| ERRγ-4-OHT-RIP140| 55.4     | 0.1     | 2    |
| ERRγ-SMRT        | 48.8     | 0.2     | 2    |
| ERRγ-4-OHT-SMRT  | 54.9     | 0.1     | 2    |

$^a$ S.D. values were calculated from replicates.

$^b$ n, number of experiments carried out.

The movement of hervolistic bonds involving the ligand are shown in red, with distances denoted in angstroms. The ligand hydrogen bonds directly to two residues, the side chain of Asp-328 and the backbone carbonyl of Tyr-327. Indirect hydrogen bonds to the ligand exist through two waters to the side chain of Arg-316 and the main chain of Leu-309. Residues denoted in blue are >4.0 Å from the ligand.

**Figure 6.** Overlay of unliganded ERRγ/RIP140 and the ERRγ-GSK4716-RIP140 complexes. The unliganded structure of ERRγ is represented as a yellow ribbon, with selected side chains depicted using cyan carbons, and its corresponding coregulator peptide is shown in green. The GSK4716 structure is shown as an orange ribbon, with side chains depicted using magenta carbons, and its corresponding coregulator peptide shown in purple. A space filling representation of the GSK4716 compound is shown with green carbon, red oxygen, and blue nitrogen atoms. The most notable movement in the main chain upon binding of compound occurs in the region just below helix 1 involving residues 246–248, although some movement of all residues N-terminal to helix 3 was observed. The volume available to the ligand in the pocket of the ERRγ-GSK4716-RIP140 complex is depicted with a semitransparent surface. This induced pocket, which has a volume of 610 Å³, resulted from a merging of the two pockets shown for the apo receptor in Fig. 2, which occurred when the salt bridge between Asp-275 and Arg-316 was disrupted. The portion of the combined pocket contributed by the apo pocket 1 remained fairly static, whereas there was significant rearrangement of the residues that lined the apo pocket 2 changing its shape.

**Figure 7.** Plot of ligand interactions in the ERRγ-GSK4716-RIP140 complex. Hydrogen bonds involving the ligand are shown in red, with distances denoted in angstroms. The ligand hydrogen bonds directly to two residues, the side chain of Asp-328 and the backbone carbonyl of Tyr-327. Indirect hydrogen bonds to the ligand exist through two waters to the side chain of Arg-316 and the main chain of Leu-309. Residues denoted in blue are >4.0 Å from the ligand.

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$^a$ S.D. values were calculated from replicates.

$^b$ n, number of experiments carried out.

The thermal sensitivity assay, the results of which are shown in Table 2. The change in transition temperature ($t_{1/2}$) of the ERRγ-LBD upon interaction with small molecule ligands and peptide fragments was followed by circular dichroism (CD, ellipticity at 222 nm). The acyl hydrazone GSK4716 increased $t_{1/2}$ compared with the unliganded receptor by 0.9 ± 0.1 °C, indicating a small increase in protein stability upon binding of the agonist ligand. The RIP140 LXXLL peptide increased $t_{1/2}$ to a greater extent (3.0 ± 0.2 °C), and the effect on protein stability was at least additive when both the peptide and acyl hydrazone were added to the LBD (increase of 4.2 ± 0.2 °C). The inverse agonist 4-OHT increased $t_{1/2}$ compared with the unliganded receptor $0.1 °C$, but unexpectedly, the effect of 4-OHT on protein stability was at least additive when both the peptide and acyl hydrazone were added to the LBD (increase of 4.2 ± 0.2 °C). The inverse agonist 4-OHT increased $t_{1/2}$ of the LBD (6.7 ± 0.1 °C). However, the effect of 4-OHT on protein stability was at least additive with the LXXLL peptide (7.1 ± <0.1 °C increase for the ternary complex versus unliganded receptor), as expected from crystal structures and peptide binding assays. The SMRT peptide increased $t_{1/2}$ by 0.5 ± 0.1 °C, but unexpectedly, the effect of this peptide was neither additive nor synergistic with 4-OHT. This indicates that 4-OHT binding alone leads to the most stable conformation of ERRγ LBD. Thermodynamically, the ternary complex formed by SMRT and 4-OHT binding to ERRγ LBD must be energetically favored, so this stabilization may be too small or does not contribute to the signal measured by this technique, under the experimental con-
discussions chosen. This may be a consequence of the thermal transitions being irreversible, and therefore analysis cannot be thermodynamically rigorous. Alternatively, there may be some energetic compensation in the formation of this ternary complex, which masks the stabilization. The thermal stability data suggest that the agonist activity of the acyl hydrazone GSK4716 is due in part to global stabilization of the receptor protein in an active conformation that permits the binding of transcriptional coregulator proteins.

**DISCUSSION**

Herein we report crystal structures for ERRγ in three distinct states: unliganded, inverse agonist-bound, and agonist-bound. Comparison of the three states reveals a significant degree of receptor plasticity, with rearrangements observed within the ligand binding pocket to accommodate ligand. X-ray crystallography has previously revealed a significant degree of protein adaptability in the binding of ligands of various size in other nuclear receptors such as the pregnenolone X receptor (24) and the liver X receptor (25). However, there is no other example where all three states have been observed crystallographically. As such, the ERRγ structures reported herein provide new insight into the mechanism of transcriptional regulation by small molecule ligands.

A smaller number of apo compared with ligand-bound NR LBD crystal structures has been reported, in part because of the stability conferred to the protein upon binding of a ligand in the hydrophobic pocket. The ERRγ LBD is sufficiently stabilized to crystallize not only without bound ligand but also in the absence of a stabilizing cofactor peptide. Comparison of apo-ERRγ in the presence and absence of the RIP140 LXXLL peptide showed that no significant conformational changes occurred in the LBD structure apart from the conformation of the surface-exposed charge clamp residues, which become ordered upon peptide binding. The stability of the apo receptor is consistent with the relatively small volume of the ligand binding pocket, which is partially filled with bulky hydrophobic residues and is thus not large enough to bind conventional steroid hormones. These data suggest that the apo-ERRγ is preorganized in an active conformation in cells such that the coregulator functions as a protein ligand upon binding to the transcription factor.

The inverse agonist structures of ERRγ with 4-OHT ± SMRT give us an insight into other possible mechanism(s) of ERRγ repression beyond those involving unusual coregulators such as RIP140. Although we do not believe that the SMRT peptide used in our current study is optimal for repression of ERRγ, it does give us an understanding how more conventional corepressors may bind in the ER/ERR family of receptors. Several lines of evidence lead us to believe that the SMRT structure presented here may be relevant beyond the fact that it bound specifically to the LBD. Huang and coworkers demonstrated in ERα that, although *in vitro* assays have not been successful at establishing the binding of the corepressors NCOR or SMRT with great affinity, they could establish the ligand-dependent binding of phage display peptides from a corepressor NR box library (26). This work also identified through mutations with ERα, that residue Leu-372 was critical for their observed peptide binding. The equivalent residue in ERRγ, Leu-294, helps to create the hydrophobic pocket (along with Met-298) in which the C-terminal end of the peptide anchors itself to the LBD by interacting with Leu-1328 of the peptide. The carboxyl of Leu-1328 also interacts with the Lys-284 forming the charge clamp seen in other corepressor structures. The position of the SMRT peptide in our ERRγ-4-OHT-SMRT complex is very close (1.35 ÅCa root mean square) to that observed in another steroid corepressor structure with an SMRT-derived peptide. Finally, the N-terminal end of the peptide is positioned within ~4.6 Å of the ligand itself, which is again consistent with other steroidal corepressor observations. Although the distance we see is too long to argue that a direct interaction between the peptide and the 4-OHT ligand exists, it does suggest that appropriate ligands could directly modulate binding of the peptide on this surface.

In the ERRγ-4-OHT structure the AF-2 helix is a position (at ~60% occupancy in our structure) that would prevent all coregulator binding. The tetrameric structure seen in the crystal complex with 4-OHT and a SMRT peptide fragment shows an interaction of the receptor with a second homodimer of itself, again blocking at least one of the sites for coregulator interaction. It is interesting to speculate if either of these two modes of peptide binding inhibition is relevant *in vivo*.

Previous structural studies have helped to rationalize the constitutive activity of unliganded ERRγ. The receptor adopts an active conformation in which AF-2 helix is oriented favorably for coactivator binding. In contrast, the classic mechanism for nuclear receptor agonism depends on a ligand to stabilize the active conformation. However, because the ERRγ AF-2 helix is already oriented to allow LXLL coregulator binding, the activation of the receptor by the agonist GSK4716 must occur by a different mechanism. Moreover, because a significant reorganization of the apo receptor is observed in the agonist-bound structure, it was not obvious that ligand binding would lead to increased receptor activity. In exploring the activation mechanism, thermal stability studies demonstrated that GSK4716 stabilizes the active receptor. Thus, a plausible mechanism of transcriptional activation could be an increase in the cellular half-life of the receptor. Although we have not explored the turnover of the receptor in cells, the concept of seeking ligands that increase protein stability *in vitro* may provide an alternative approach to the discovery of agonists for constitutively active orphan nuclear receptors.

Prior to solving the ERRγ-GSK4716-RIP140 cocystal structure, we had anticipated that the phenolic group on the ligand would be bound to Glu-275 and Arg-316 as has been observed with other ER and ERR ligands. However, molecular modeling showed that there was not sufficient room in the binding pocket to accommodate GSK4716 without rotation of Phe-435 into a conformation that would displace the AF-2 helix, as seen with the ERRγ inverse agonists 4-OHT and diethylstilbestrol. Remarkably, the cocystal structure revealed that GSK4716 forces a rotation of both Glu-275 and Arg-316 that allows coordinates and structure factors have been deposited into the Protein Data Bank with the following accession ID numbers, 2GP7 (ERRγ apo structure), 2GPO (ERRγ-RIP140 complex), 2GPX (ERRγ-GSK4716-RIP140 complex), 2GPU (ERRγ-4-OHT complex), and 2GPV (ERRγ-4-OHT-SMRT complex).
access of the ligand to another pocket in the ligand binding domain. The volume of the single combined pocket is 610 Å³, which is large enough to accommodate the acyl hydrazone ligand without requiring displacement of the AF-2 helix.

A dimer is present in the asymmetric unit of the ERRγ/GSK4716/RIP140 complex. However, only one of the molecules in the dimer appears to contain a ligand. Superposition of the two LBDs within the structure shows no obvious movements of the main or side chains, which extend toward the helix 10 dimer interface. This suggests that binding to one side of the dimer does not influence the other. Each of the two LBDs, however, appears to sit in different environments with respect to other protein molecules in the crystal. The LBD, which contains the ligand, has no crystal contacts on helix 1 and makes a minor contact containing one hydrogen bond to a neighboring molecule at residue 250, just below the region that loops out to help form the ligand pocket. The unliganded LBD has an extensive series of contacts down the face of helix 1 extending to residue 243 and a second series of contacts involving residues 250–256 prior to the turn that initiates helix 3. As described previously, the entire N-terminal region prior to helix 3 shifts in the agonist-bound LBD relative to the other structures in this study. These crystal contacts may be stabilizing the unliganded conformation of the second LBD. This could have the effect of shifting the equilibrium of this LBD to favor the unbound state conformation of the second LBD. This could have the effect of shifting the equilibrium of this LBD to favor the unbound state conformation of the second LBD. This could have the effect of shifting the equilibrium of this LBD to favor the unbound state conformation of the second LBD. The binding mode of GSK4716 explains its selectivity for binding to ERRγ over the classic ERs. GSK4716 is a submicromolar ERRγ ligand with no affinity for ERα or ERβ at concentrations up to 50 μM. The phenolic ring of GSK4716 forms a hydrogen bond with Asp-328 near the surface of the receptor. In ERα this residue correlates to Pro-406, which would not be able to interact in the same manner with the phenolic group. Although the ligand interacts with the conventional phenol binding residue Arg-316, it does so through one of two bound water molecules that bridge to the carbonyl group of the acyl hydrazone. The second conventional phenol binding residue Glu-275 is rotated into a conformation where it interacts with Glu-247 in ERRγ. In ERα, the corresponding residue is Pro-325, which would be unable to stabilize the rotated conformation of the glutamic acid. Thus, it is likely that rotation of the phenol binding residues in the classic ERs, which permit access to the secondary binding pocket, is less energetically favorable. Combined with the lack of a phenol binding site to accommodate the alternative binding mode, these differences explain the binding selectivity observed for GSK4716.

Peptide interaction experiments indicated that the optimal ERRγ interaction partner surveyed was RIP140 (residues 366–390). RIP140 is an unconventional nuclear receptor coregulator. Although it binds using a coactivator-like LXXLX NR box sequence, the protein has corepressor-like activity on transcription factors. In our experiments we observed that increased recruitment of the RIP140 LXXLX peptide fragment by fluorescence resonance energy transfer assay correlated with transcriptional activation in the cell-based assays. The ERRγ-RIP140 (366–390) crystal structure has AF-2 helix in the agonist conformation, and the peptide fragment, which includes an LXXL motif, binds to the receptor in a manner largely identical to that observed with the coactivator NR box peptide SRC1 (residues 686–700). These data suggest that ERRγ binds to RIP140 with the receptor in the agonist conformation setting up a situation in cells where there is a dynamic competition between RIP140 and coactivators such as SRC1 and PGC-1α. The role of ERRγ may be to function as a docking site for these coregulators in the promoters of target genes rather than a hormone-regulated receptor like its cousins the classic ERs. In this paradigm it is remarkable that we can identify synthetic small molecule ligands that can further activate transcription through ERRγ.

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