Crystal Structure of Human Estrogen-related Receptor α in Complex with a Synthetic Inverse Agonist Reveals Its Novel Molecular Mechanism*

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Inverse agonists of the constitutively active human estrogen-related receptor α (ERRα, NR3B1) are of potential interest for several disease indications (e.g. breast cancer, metabolic diseases, or osteoporosis). ERRs is constitutively active, because its ligand binding pocket (LBP) is practically filled with side chains (in particular with Phe328, which is replaced by Ala in ERR pocket(LBP)is practically filled with side chains (in particular with disease indications (e.g. breast cancer, metabolic diseases, or osteoporosis)). We present here the crystal structure of the ligand binding domain of ERRα (containing the mutation C325S) in complex with the inverse agonist cyclohexylmethyl-(1-p-tolyl-1H-indol-3-ylmethyl)-amine (compound 1a), to a resolution of 2.3 Å. The structure reveals the dramatic multiple conformational changes in the LBP, which create the necessary space for the ligand. As a consequence of the new side chain conformation of Phe328 (on helix H3), Phe510 (H12) has to move away, and thus the activation helix H12 is displaced from its agonist position. This is a novel mechanism of H12 inactivation, different from ERRγ, estrogen receptor (ER) α, and ERβ. H12 binds (with a surprising binding mode) in the coactivator groove of its ligand binding domain, at a similar place as a coactivator peptide. This is in contrast to ERRγ but resembles the situation for ERRα (raloxifene or 4-hydroxytamoxifen complexes). Our results explain the novel molecular mechanism of an inverse agonist for ERRα and provide the basis for rational drug design to obtain isotype-specific inverse agonists of this potential new drug target. Despite a practically filled LBP, the finding that a suitable ligand can induce an opening of the cavity also has broad implications for other orphan nuclear hormone receptors (e.g. the NGFI-B subfamily).

In mammals, the nuclear hormone receptor (NR)2 superfamily consists of 48 transcription factors that control essential developmental and physiological pathways (1). Although the transcriptional activity of NRs is often regulated by specific ligands, several members of the superfamily have no known natural ligands and are therefore referred to as orphan NRs (2). Estrogen-related receptor α (ERRα; NR3B1) was the first orphan NR to be identified on the basis of its similarity with estrogen receptor α (ERα; NR3A1) (3). ERRα and its relatives ERRβ (NR3B2) and ERRγ (NR3B3) form a small family of orphan NRs that are evolutionarily related to the estrogen receptors ERα and ERβ. ERRs preferentially bind to DNA sites composed of a single half-site preceded by three nucleotides with the consensus sequence TNAAGGTCA, referred to as an ERR-response element. It has been shown that ERRα also efficiently binds to estrogen-response elements and that these receptors share common target genes (4). This observation was further supported by studies demonstrating cross-talk between the ER and ERR pathways (reviewed in Ref. 5). Several studies have highlighted ERRα as a main player in mitochondrial biogenesis and oxidative metabolism (6–8), suggesting that ERRα could be used for therapeutic intervention in diabetes or metabolic diseases. In addition to this central role in metabolism, ERRα is also now accepted as an emerging target in cancer (9, 10). Finally, few papers have suggested a role of ERRs in bone metabolism (reviewed in Ref. 11). The importance of ERRs as a drug target has been recently reviewed (12), which further re-emphasizes the urge for new synthetic ligands in the ERR subfamily.

Despite their overall sequence similarity with the ERs, ERRs seem to regulate transcription in the absence of known natural agonist ligands. The presence of a phenylalanine residue (Phe328 on helix H3) in the ligand binding pocket (LBP) has been found to be essential for the constitutive activity of ERRα, because its mutation to alanine abolishes constitutive activity (13). The x-ray structure of apoERRα, in complex with a coactivator peptide containing the L3 site of peroxisome proliferator-activated receptor coactivator-1α (PGC-1α), had revealed that the LBP is practically filled with side chains (14). The unoccupied volume was found to be only ~100 Å3. It was predicted that a ligand with an equivalent of more than ~4–5 carbon atoms could only bind if Phe328 would drastically change its conformation. This would also require a displacement of Phe510 and thus of H12, making the ligand an inverse agonist. The term “inverse agonist” (instead of “antagonist”) is used because such a ligand would display an intrinsic activity, namely an inhibition of the constitutively active ERRα. In contrast a neutral antago-
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nist does not have an intrinsic activity by itself, it just counteracts the binding of an agonist.

Indeed, searches for ERRα ligands have so far mostly identified inverse agonists. Few synthetic ligands, classically linked to the ER pathway, have been shown to modulate ERRα activity, namely 4-hydroxytamoxifen (4-OHT) and diethylstilbestrol (DES) (reviewed in Ref. 15). In addition, a new synthetic ligand acting as an inverse agonist has been identified for ERRα. It was shown that it acts at submicromolar activities in a cell-based assay and that this compound inhibits ERRα induction of an ERRα target gene, monoamine oxidase B (16, 17).

For ERRγ, the crystal structures for the complexes of its ligand binding domain (LBD) with the inverse agonists DES and 4-OHT (18), a 4-OHT derivative (19), and 4-OHT together with a corepressor peptide (20) have been published. The cavity volume of the LBP for unliganded ERRγ was reported as 220 Å³ (21), which is small but still considerably larger than the 100 Å³ described for apoERRα. This difference is mainly explained by the substitution of Phe328 (ERRα) with Ala350 (ERRγ). In all reported complexes of ERRγ with inverse agonists, the activation helix H12 was found to be completely dissociated from the LBD body (18–20) and also partially disordered (20). The ERRγ structures are thus in contrast to selective ER modulator (SERM) complexes for ERRα, where binding of raloxifene or 4-hydroxytamoxifen revealed H12 in the coactivator groove. In the latter ERRα complexes, accompanying structural adaptations of the C-terminal end of H11 and of the H11/H12 loop (22, 23) were observed. For ERRβ LBD complexed with the full antagonist ICI 164,384 (ICI), H12 was not visible because of high mobility (24).

For ERRα it was difficult to predict the exact details of how (and whether at all) a ligand (most likely acting as an inverse agonist) would bind in the LBP, because of the multiple conformational changes required to create the necessary space and interactions. Similarly, it was not clear what exact consequences ligand binding would have on H12, except that it would probably be displaced from the agonist position.

Here we report the crystal structure of the human ERRα LBD in complex with the synthetic inverse agonist cyclohexylmethyl-(1-p-tolyl-1H-indol-3-ylmethyl)-amine (Fig. 1C, compound 1a), at 2.3 Å resolution. Compound 1a is a derivative of compound 1b (Fig. 1C), which lacks the methyl group and originally was discovered by high throughput screening. We have introduced the mutation C325S in ERRα in order to reduce biochemical instability problems during purification and crystallization, associated with cysteine oxidation. Isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) measurements have demonstrated that there are no significant differences in the thermodynamic binding parameters between wild type ERRα LBD and the C325S mutant for compound 1a (or 1b). Also binding studies by NMR spectroscopy of compound 1b to wild type ERRα in solution (with and without a spin label attached to Cys325) revealed no violations of the observed relaxation effects with the crystal structure of the compound 1a complex.

Our results explain the novel molecular mechanism of an inverse agonist for ERRα and provide the basis for rational drug design to obtain selective inverse agonists of this potential new drug target.

MATERIALS AND METHODS

Protein Cloning, Expression, and Purification—The mutation C325S was introduced into the plasmid pX1392 (wild type ERRα LBD, amino acids 290–519, numbering according to Swiss-Prot P11474 (14)) by site-specific mutagenesis using the QuikChange mutagenesis kit from Stratagene. The correctness of the construction was verified by DNA sequence analysis, and the correct clone was called pX1392b. Generation of recombinant baculovirus by transfection with BacPAK8™, plaque cloning, and amplification was done as described (14). For production of ERRα(C325S) LBD-expressing cells, 1-liter shake flask cultures with Sf9 cells adapted to SF900 II (Invitrogen) at a density of 1.5 × 10⁶ c/ml were infected with baculovirus at a multiplicity of infection = 1 and were cultured for 3 days at 28 °C and 90 rpm, as described previously (14). Harvest of the cells was carried out as described (14). SDS-PAGE analysis (Coomassie staining and Western blotting with anti-penta-His antibody from Qiagen) revealed good and soluble expression of ERRα(C325S) LBD. The purification of the frozen Sf9 cell pellets was done by three-step chromatography as described previously (14). The purified ERRα LBD protein in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM DTT was concentrated to 10.4 mg/ml prior to crystallization. The resulting protein was estimated to be >95% pure and homogeneous by SDS-PAGE and reverse phase high pressure liquid chromatography coupled to mass spectrometry. The measured molecular mass of 27,025.3 Da of the protein corresponded to the acetylated des-Met form of the ERRα (290–519; C325S) LBD.

Crystallization, Data Collection, and Structure Determination—Compounds 1a and 1b were synthesized in-house. For cocrystallization, a 5:1 stoichiometric excess of compound 1a from a 100 mM stock solution in Me2SO was added to the protein solution. Crystals were obtained at 4 °C by the vapor diffusion method in 1-μl hanging drops containing equal volumes of protein (10.4 mg/ml ERRα(C325S) LBD, 100 mM NaCl, 5 mM DTT, 50 mM Tris-HCl, pH 7.5) and crystallization buffer (0.2 M MgCl₂, 12% PEG400, 0.1 M HEPES, pH 7.5). The initial crystallization hit was obtained with Crystal Screen I from Hampton Research. Crystals were directly mounted in cryoloops and flash-frozen in the nitrogen stream. Diffraction data at 100 K were collected at the Swiss Light Source (beamline X10SA), using a Marresearch CCD detector and an incident monochromatic x-ray beam with 0.8 Å wavelength. Raw diffraction data were processed and scaled with the HKL program suite version 1.96.1 (26). The estimated B-factor by Wilson plot analysis is 48.6 Å². The structure was determined by molecular replacement with MOLREP (27, 28) using as starting model the coordinates of ERRα LBD (Protein Data Bank access code 1XB7) refined to 2.5 Å resolution (14), with H12 removed. The program REFMAC version 5.0 (28, 29) was used for refinement. Bulk solvent correction, an initial anisotropic B-factor correction, and restrained isotropic atomic B-factor refinement were applied. The refinement target was the maximum likelihood target using amplitudes. No σ cut-off was applied on the structure factor amplitudes. Cross-validation was used throughout
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![Diagram A](image1.png)

![Diagram B](image2.png)

![Diagram C](image3.png)

![Diagram D](image4.png)

**FIGURE 1.** Overall crystal structure of ERRα LBD in complex with cyclohexylmethyl-(1-p-tolyl-1H-indol-3-ylmethyl)-amine and comparison with apoERRα-PGC-1α. A, ribbon drawing showing the complex between apoERRα LBD (color ramped from the N terminus in blue to the C terminus in red) and the PGC-1α peptide (α-helix in white). The broken line indicates a flexible and thus unmodeled loop of ERRα. The coactivator peptide is bound to the AF-2 site and makes stabilizing interactions with H12, which are possible because of the "agonist position" of H12. H12 adopts this conformation without a ligand bound in the LBP, which explains the constitutive activity of ERRα. B, ribbon drawing showing the complex between ERRα LBD and the inverse agonist cyclohexylmethyl-(1-p-tolyl-1H-indol-3-ylmethyl)-amine (CPK-model with carbons in white and nitrogens in blue). The presence of an inverse agonist in the LBP leads to an unwinding of the last turn of H11 and a movement of the N-terminal part of H3. H12 adopts a dramatically new position, by binding to the AF-2 site (at a similar position as the PGC-1α peptide). This position of H12 directly interferes with coactivator binding and explains the inverse agonism of the ligand. C, chemical structures of compound 1a (cyclohexylmethyl-(1-p-tolyl-1H-indol-3-ylmethyl)-amine) and the parent compound 1b. D, compound 1a fitted into the 2Fo - Fc electron density map. Compounds 1a and 1b have IC50 values of 190 and 700 nM to the ERRα LBD, as determined by the FRET assay. Figs. 1, A, B, and D, and 2 and 3 were generated by PyMOL (41).

refinement using a test set comprising 5.1% (1056) of the unique reflections. Water molecules were identified with the program ARP/wARP (28, 30) and selected based on difference peak height (greater than 3.0σ) and distance criteria. Water molecules with temperature factors greater than 60 Å2 were rejected. The program O version 7.0 (31) was used for model rebuilding, and the quality of the final refined model was assessed with the programs PROCHECK version 3.3 (32) and REFMAC version 5.0 (28, 29). Crystal data, data collection, and refinement statistics are shown in Table 1.

*Isothermal Titration Calorimetry, Differential Scanning Calorimetry, and Fluorescence Energy Transfer Measurements*—The ITC experiments were performed using a Microcal VP-ITC instrument (Microcals, Inc., Northampton, MA). The sample cell of the calorimeter was loaded with ERRα LBD (wild type and C325S mutant at 40 and 30 μM) in 50 mM Tris, pH 7.5, 100 mM NaCl, and 0.5 mM TCEP. The syringe was loaded with compound 1b (400 μM) in the same buffer. All solutions were degassed for 10 min. Titrations were performed at 25 °C with injection volumes of 8 μl and a spacing of 240 s. The base line was set to zero assuming that the final injections of each titration represent only the heat of dilution. The data were fit using a one-site binding model available in the Origin ITC data analysis software (version 7.0). DSC analysis was performed on buffer solutions (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM TCEP) containing the ERRα LBD-compound 1a complex (1:10 molar ratio). The ERRα LBD concentration was 25 μM (both for wild type or the C325S mutant), and 300 μl of protein complex solution were injected. DSC scans were obtained using a MicroCal VP-capDSC system (Microcal, LLC, Northampton, MA) at a scan rate of 200 °C/h. The fluorescence energy transfer (FRET) measurements were conducted as time-resolved measurements (TR-FRET) in a miniaturized 1536 well plate format. Typically, 100 nl of compound solution (2 mM in 90% Me3SO) were transferred to the assay plate. 3 μl of assay buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.05% bovine serum albumin, 5 mM DTT, 0.1% Pluronics F-127) containing LANCE EU-W1024-labeled anti-His6 antibody (PerkinElmer Life Sciences; 1 nM final assay concentration) were added. 1 μl of His-ERRα LBD (50 nM final assay concentration) was added, and the plates were left for incubation at 20 °C for 30 min. As FRET acceptor, 1 μl of an N-terminally Cy5-labeled PGC-1α derived peptide (Cy5-RPCSELLKYLTLT, 50 nM final assay concentration) was added. After 20 min of incubation the TR-FRET readout was performed on an Envision 2102 multilabel reader (PerkinElmer Life Sciences) with an excitation at 350 nm, first emission (donor) at 615 nm, and second emission (acceptor) at 665 nm (30 flashes, 100-μs delay time). The readout was calculated according to X = donor/acceptor × 1000. Data were fitted with XLfit4 after plate normalization of the derived data.

*NMR Spectroscopy*—Ligand binding studies were performed with wild type and spin-labeled ERRα LBD. The spin-labeled protein was obtained by addition of a maleimide-substituted tetramethyl pyrroline N-oxide (TEMPO) paramagnetic tag (Aldrich) that reacts selectively with the only cysteine residue present (Cys325). Selective reaction was completed within minutes at room temperature prior to the measurement. T1, relaxation and water-LOGSY experiments were performed at 600 MHz using a room temperature probe.
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**TABLE 1**
Crystalllographic summary

| Diffraction data          | Space group | H3 (R3, hexagonal setting) |
|---------------------------|-------------|-----------------------------|
| Complexes                |             | 2 per asymmetric unit       |
| Unit cell dimensions     | \(a = b \approx 96.5\) \(\text{Å}, c = 134.6\) \(\text{Å}\) |
| Source                    |             | SLS XI05A                   |
| Wavelength                |             | 0.8000 \(\text{Å}\)         |
| Resolution range          |             | 20.0 to 2.3 \(\text{Å}\)    |
| No. of unique reflections |             | 20,542                      |
| No. of observations       |             | 256,183                     |
| \(|I/|\sigma(I)|\)      |             | 39.1 (4.8)                  |
| \(R\_\text{free}}\) on intensities\(^a\) |             | 0.068 (0.24)                |
| Completeness              |             | 99.5% (99.9%)               |

| Refinement               | Resolution range | 20.0 to 2.3 \(\text{Å}\) |
|--------------------------|------------------|---------------------------|
| \(R\_\text{cryst}}\)     | 0.219            |
| \(R\_\text{free}}\)      | 0.281            |
| Protein atoms (chains A/B) | 1694/1694      |
| Ligand atoms (chains L/M)   | 25/25           |
| Water molecules            | 236             |
| Average \(R\_\text{factor}}\) for protein | 42.7 \(\text{Å}\)  |
| Average \(R\_\text{factor}}\) for ligand | 31.3 \(\text{Å}\) |
| r.m.s.d. from target values | Bond lengths | 0.008 \(\text{Å}\) |
| Bond angles               | 1.13            |
| Ramachandran plot (chains A/B) | Most favorable regions | 93.8/92.7% |
|                          | Allowed regions  | 5.7/7.3%                   |
|                          | Genetically allowed regions | 0.5/0.0%                   |

\(^a\) \(R\_\text{free}}\) = \(|F_o\) – \(|F_c|\)/\(|F_o|\), where \(|F_o|\) and \(|F_c|\) are observed and calculated structure factors; \(R\_\text{free}}\) is calculated for a randomly chosen 5% of reflections, and \(R\_\text{cryst}}\) is calculated for the remaining 95% of reflections.

RESULTS AND DISCUSSION

The Overall Structure of the Complex between ERR\(\alpha\) LBD and the Inverse Agonist Compound 1a Reveals H12 in a Position Similar to PGC-1\(\alpha\)–We have solved the crystal structure of ERR\(\alpha\) LBD (containing the mutation C325S) in complex with the inverse agonist cyclohexylmethyl-(1-p-tolyl-1H-indol-3-ylmethyl)-amine (compound 1a) (Fig. 1C), at 2.3 \(\text{Å}\) resolution (space group H3). Compound 1a is a derivative of compound 1b, which originally was discovered by high throughput screening. The asymmetric unit contains a homodimer of ERR\(\alpha\) complexes, i.e. ligand binding did not interfere with dimer formation. The results of the crystallographic refinement are summarized in Table 1. In general the electron density for the ERR\(\alpha\) LBD is well defined, except for the His tag, residues Pro\(^{309}\)–His\(^{317}\) (H2/H3 loop), residues Arg\(^{462}\)–Glu\(^{570}\) (H9/H10 loop), and the C-terminal residues Met\(^{518}\)–Asp\(^{519}\) (all of which were not modeled). The protein part of the refined model consists of the PreScission\(^\text{TM}\) site (LEVLFQGP) followed by amino acids Val\(^{299}\)–Met\(^{308}\), Leu\(^{318}\)–Gly\(^{466}\), and Arg\(^{471}\)–Met\(^{517}\) of the ERR\(\alpha\) LBD. The refined model also contains 236 water molecules and two compound 1a molecules. The two complexes in the asymmetric unit are very similar, with a root mean square deviation (r.m.s.d.) of 0.13 \(\text{Å}\) for 210 C\(^{\text{\alpha}}\) atoms of residues 291–308, 332–461, and 471–498.

A comparison of apoERR\(\alpha\) (bound to a PGC-1\(\alpha\) peptide (14)) with the inverse agonist complex (Fig. 1) revealed no major conformational changes for the main chain atoms from H1 to the middle of H11 (r.m.s.d. of 0.55 \(\text{Å}\) for 210 C\(^{\text{\alpha}}\) atoms as above), with the exception of H3. The N-terminal part of H3 moves away from the LBP, in order to create necessary space for the ligand (Fig. 1). In particular the C\(^{\text{\alpha}}\)’s of Val\(^{221}\) and Leu\(^{324}\) move by 4.3 and 3.2 \(\text{Å}\), respectively. Important structural perturba-

tions of the main chain due to compound 1a binding also occur at the C terminus of H11 (unwinding of the last turn of H11, i.e. after Lys\(^{499}\)), the H11/H12 loop (reorganization of its conformation, so that Pro\(^{505}\) is now at the beginning of H12), and H12 (new location in the coactivator binding groove) (Fig. 1). In the apoERR\(\alpha\) complex with PGC-1\(\alpha\) (14), H12 is formed between His\(^{507}\) and Ala\(^{516}\) (i.e. Pro\(^{505}\) and Met\(^{506}\) are in the H11/H12 loop), whereas in the inverse agonist complex H12 already starts at Pro\(^{505}\) and is visible until Met\(^{517}\). In the apoERR\(\alpha\) structure, the PGC-1\(\alpha\) peptide (which adopts an \(\alpha\)-helical conformation) utilizes two Leu side chains (Leu\(^{210}\) and Leu\(^{214}\)) of its inverted LXXLL-motif to make hydrophobic interactions in the coactivator groove. Unexpectedly, similar interactions are made by two Met side chains (Met\(^{313}\) and Met\(^{517}\)) of H12 in the compound 1a complex. The hydrogen bond made between Lys\(^{340}\)(H3) and the PGC-1\(\alpha\) main chain (contributing to the “charge clamp” interaction) is replaced by a hydrogen bond between Lys\(^{340}\) and the carbonyl oxygen of Ala\(^{516}\)(H12). H12 in the compound 1a complex is making further stabilizing interactions via a salt bridge between the side chains of Lys\(^{508}\)(H12) and Asp\(^{579}\)(H3) and via a hydrogen bond between the carbonyl oxygen of Met\(^{506}\)(H12) and the side chain of Gln\(^{340}\)(H4). Finally, in the inverse agonist complex Leu\(^{339}\)(H12) is located in the same hydrophobic pocket (with Trp\(^{361}\) at the bottom) as Met\(^{513}\)(H12) in the apoERR\(\alpha\) structure. In the crystal lattice, Phe\(^{510}\) makes an aromatic stacking interaction with Phe\(^{510}\)(of a neighboring molecule, but these packing interactions seem to be of less importance than the interactions in the coactivator groove itself (described above).

The finding that for the ERR\(\alpha\)-compound 1a complex H12 adopts a well defined position in the coactivator groove is in contrast to ERR\(\gamma\), where the binding of diethylstilbestrol or 4-hydroxytamoxifen led to complete dissociation of H12 from the LBD (18–20). On the other hand, the ERR\(\alpha\) structure resembles the situation for ERR\(\gamma\), where binding of the SERMs raloxifene or 4-hydroxytamoxifen revealed H12 in the coactivator groove, with accompanying structural adaptations of the C-terminal end of H11 and of the H11/H12 loop (22, 23). For ERR\(\gamma\) LBD complexed with the full antagonist ICI, H12 was not visible because of its high mobility (24).

Compound 1a Binds in the LBP of ERR\(\alpha\) by Displacing Phe\(^{328}\)(H3) and Phe\(^{510}\)(H12); the Novel Mechanism of an ERR\(\alpha\) Inverse Agonist—The empty cavity of the LBP in apoERR\(\alpha\) has a volume of only \(\sim 100\) \(\text{Å}^3\) (14), so that multiple structural adaptations are required to enable ligand binding. In apoERR\(\alpha\) the aromatic side chains of Phe\(^{328}\)(H3), Phe\(^{495}\)(H11), and Phe\(^{510}\)(H12) cluster (Fig. 2A) and in particular the presence of Phe\(^{328}\) (which is replaced by Ala for ERR\(\beta\) and ERR\(\gamma\)) leads to an almost complete occupation of the LBP with side chains. Compound 1a binding induces a new side chain conformation for Phe\(^{328}\)(H3) and as a consequence a displacement of Phe\(^{510}\) (to avoid a steric clash with Phe\(^{328}\)), which triggers a dramatic movement of H12 (Fig. 2B). Phe\(^{495}\)(H11), on the other hand, has almost the same position in both structures. In the compound 1a complex, Phe\(^{495}\) makes an aromatic stacking interaction with Phe\(^{328}\), and they form together a hydrophobic lid on top of the ligand (Fig. 2B). In addition to the reorientation of Phe\(^{328}\), also the N-terminal part of H3 moves away from the...
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A FIGURE 2. The movement of H12 is triggered by a conformational change of Phe328. Color coding is as in Fig. 1, but with view zoomed in from the left and selected side chains depicted as stick models. A, aromatic side chains of Phe328(H3), Phe395(H11), and Phe310(H12) cluster when no ligand is bound in the LBP of ERRα. In particular, the presence of Phe328 leads to an almost complete filling of the LBP by side chains. B, binding of the inverse agonist compound 1a (ball-and-stick-model, carbons in white and nitrogens in blue) in the LBP of ERRα requires a new side chain conformation of Phe328(H3). As a consequence also Phe310(H12) and thus H12 are displaced. H12 adopts a new position by binding to the AF-2 site (and thus competes directly with the binding of PGC-1α). The diagram is programmed for stereo viewing.

In order to create space for the ligand via removal of Val321 and Leu324 (Fig. 3B). Taken together, Phe510 could be regarded as a sensor that transduces the conformational state of Phe328 (which depends on the ligand occupation of the LBP) into a position of H12 (turning the transcriptional activity of ERRα “on” or “off” (Fig. 1)).

The Amino Nitrogen of Compound 1a Makes a Salt Bridge with Glu331—The binding pocket of compound 1a is delineated (cutoff 4.2 Å) by amino acid residues from H3 (Val321, Leu324, Leu327, Phe328, Glu331), H5 (Met362, Leu365, Val369, Val396), the β-sheet (Phe382, Leu386), H6 (Ala396), the H6/H7 loop (Leu398), H7 (Leu405), H11 (Val501, Phe505, Val508), and the H11/H12 loop (Leu506) (Fig. 3A). All residues in the LBP have well defined electron density. The amino nitrogen of compound 1a makes a direct salt bridge and a water-mediated hydrogen bond interaction with Glu331 (Fig. 3A). The latter water molecule is well ordered (B-factor = 25 Å²) and is further stabilized by a hydrogen bond with the carbonyl oxygen of Phe382. Glu331 of ERRα corresponds to Glu331 of ERα, for which it forms e.g. a hydrogen bond with the hydroxyl of 4-OHT (23). Importantly, the side chain of Glu331 reorients toward the ligand in order to form interactions with the amino nitrogen (Fig. 3B). This reorientation is made possible because of the side chain movement of Phe328 (Fig. 3B), which creates space for Glu331 (and the ligand). The cyclohexyl moiety of compound 1a is deeply embedded inside ERRα in a mainly hydrophobic pocket. There is some space left for substituents on the cyclohexyl pointing in the direction toward Arg372. The indole moiety of compound 1a is nicely fitting in a hydrophobic pocket, which was occupied by Leu324 in the apoERRα structure (Fig. 3B). Leu324 now contributes to one side of the indole pocket. Finally, the tolyl group is the moiety that is closest to the solvent, with the methyl group at the entrance of the LBP. The carbonyl oxygens of Ala396 and Gly397 on the H6/H7 loop are potential targets to obtain additional hydrogen bond interactions.

In summary, the LBP has adapted via multiple conformational changes to the presence of compound 1a. The complementarity of fit in the hydrophobic regions is good, and the salt bridge interaction with Glu331 is likely to contribute substantially to the binding affinity.

ITC Measurements Reveal That Binding Is Enthalpically Driven—ITC measurements have revealed that the binding of compound 1b to wild type ERRα LBD is enthalpically driven with ΔH° = −12.7 kcal/mol (Fig. 4A). A negative enthalpic contribution is compatible with the formation of an important salt bridge (if desolvation of the partners is not too unfavorable). Of course, because ΔH° is composed of many contributions, it is not possible to strictly deduce the formation of a salt bridge. The entropic contribution to binding is unfavorable (−TS° = 4.6 kcal/mol). This is evidence for the hypothesis that the apo-form is more flexible than the ligand-bound form and that H12 does not become more disordered upon inverse agonist binding. Indeed the x-ray structure shows a defined position of H12 in the coactivator groove. In addition, the unfavorable entropic term indicates that desolvation (e.g. for the salt bridge partners) does not play a dominant role. Importantly the Kd values, as well as the separate enthalpic and entropic contributions, are very similar for ERRα wild type and the C325S mutant (Fig. 4) (Kd values of 770 and 930 nM, respectively). This indicates that the binding mode has not been modified by the introduction of the mutation C325S. DSC measurements (data not shown), comparing compound 1a binding to ERRα wild type and the C325S mutant, also confirmed very similar affinities (ΔTm values of −1.7–1.8 °C in both cases). The IC50 value for compound 1b binding as determined in the FRET
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FIGURE 3. Details of the binding mode of compound 1a in the LBP of ERRα and comparison with apoERRα. A, LBP of ERRα (carbon, yellow; oxygen, red; nitrogen, blue) with compound 1a bound (carbon, cyan). The amino nitrogen of compound 1a makes a direct salt bridge and a water-mediated hydrogen bond interaction with Glu331 (the latter residue corresponds to Glu353 for ERα). All other interactions are hydrophobic, mainly with the side chains of Phe428, Phe422, Leu498, Phe499, and Leu500 (the contacts with Val321 and Leu324 are not shown because of clipping). B, superposition of the LBPs of apoERRα (carbon, white) and the complex ERRα-compound 1a (coloring as in A). The main structural changes upon binding of the inverse agonist occur for Leu324 (clash with the indole moiety), Val321 (clash with tolyl moiety), and Phe328 (clash with the linker between cyclohexyl and indole moieties). The latter residue corresponds to Glu353 for ERα.

FIGURE 4. ITC measurements of ligand binding to wild type ERRα LBD and the C325S mutant. A, calorimetric titration of compound 1b into a sample cell containing wild type ERRα LBD. The titration was performed at 25 °C in 50 mM Tris, pH 7.5, 100 mM NaCl, and 0.5 mM TCEP as described under “Experimental Procedures.” The data were fit with a one-site binding model to obtain a ΔH° of −12.7 kcal/mol and −TΔS° of 4.3 kcal/mol. B, calorimetric titration of compound 1b into a sample cell containing the C325S mutant ERRα LBD. The data were fit with a one-site binding model to obtain a ΔH° of −13.0 kcal/mol and −TΔS° of 4.7 kcal/mol, i.e. values similar as for wild type. The unfavorable contribution from −TΔS° is evidence for the hypothesis that the apo-form is more flexible than the ligand-bound form and that H12 does not become more disordered upon inverse agonist binding.

Because the expected LBP is close to Cys325 (the only cysteine residue of ERRα LBD), we tried to obtain spatial information utilizing site-specific spin labeling in conjunction with T1p relaxation measurements (34). TEMPO-labeled ERRα was obtained after reaction with maleimide TEMPO, which reacts specifically with freely accessible cysteines. Significant relaxation effects induced by the paramagnetic center attached to Cys325 were measured on the individual resonances of the ligand (Fig. 5, C and D). Strong effects were observed for the resonances of the indole and phenyl groups, and weak effects were observed for the aliphatic ring, indicating that the ligand binds in proximity of Cys325 (with the cyclohexyl ring farthest away). The crystal structure of the compound 1a com-
For ERRγ, DES- and 4-OHT-mediated antagonism is caused by the rotation of the side chain of Phe435(H11), which upon ligand binding flips out of the LBP and sterically interferes with H12 in the agonist position (18–20). In contrast, for ERRα the side chain of Phe495 (which corresponds to Phe435 of ERRγ) practically does not move upon ligand binding (Fig. 3B). Compound 1a does not make a steric clash with Phe495, rather there are favorable interactions between this side chain and the ligand. For ERRα, it is the new side chain conformation of Phe328 that acts as a trigger for H12 displacement, removing it from its agonist position (Fig. 2). For ERRγ, the corresponding residue Ala272 does not have to move, because its small side chain does not lead to steric clashes with DES or 4-OHT. Docking shows also that compound 1a could fit in the ERRγ LBP without movement of Ala272 (the N-terminal part of H3 would have to bend away from the LBP similarly as for ERRα). On the other hand, the hydrophobic lid generated by Phe328 and Phe495 of ERRα on top of compound 1a (Fig. 3A) is not possible for ERRγ. This leads to the prediction that compound 1a should bind with a reduced affinity to ERRγ (and ERRβ), compared with ERRα.

Taken together, a comparison of the inverse agonist complexes of ERRα and ERRγ reveals different mechanisms of action leading to H12 displacement. For ERRα the ligand (compound 1a) displaces Phe328(H3) and thus indirectly Phe510(H12), whereas for ERRγ the ligands (DES and 4-OHT) displace Phe435(H11) and thus indirectly Leu545 and Phe450(H12). The mechanism of ERRα inactivation by compound 1a is also different from the “active” AF2 antagonists and SERMs of ERα or ERβ (where e.g. the bulky extensions of raloxifene, 4-OHT, or ICI directly displace H12) or from the “passive” AF2 antagonists of ERβ (18–20, 22–24, 35, 36).

**Differences in H12 Positions and Interactions for the Inverse Agonist Complexes of ERRα, ERRγ, and ERβ**—In the crystal structure of the ERRα–compound 1a complex H12 adopts a well defined position in the coactivator groove of its LBD. This is in contrast to ERRγ, where the binding of DES or 4-OHT led to complete dissociation of H12 from the LBD (18–20). This dissociation was explained by the absence of an LXXLL “coactivator” motif in H12 of ERRγ (18). By comparison, H12 of ERRα does contain an LXXLL motif, for which Leu536 and Leu540, i.e. the first and last Leu of the motif, mediate anchoring of H12 in the coactivator cleft for the raloxifene and 4-OHT complexes (22, 23). For ERRγ, the corresponding residues Met546 and Phe450 were thought not to allow similar stabilizing interactions of H12 with the coactivator groove (18), and thus to be an explanation for H12 dissociation. In contradiction to this hypothesis, ERRα has the same residues as ERRγ in the above motif, namely Met506 and Phe510, but nevertheless displays H12 in the coactivator cleft of its LBD for the compound 1a complex. Actually, there is no sequence difference between ERRα and ERRγ in the whole region between the ERRα residues Lys499 (C terminus of H11) and Ala516 (close to C terminus of H12). The first difference occurs at the C terminus of H12, with Met517 of ERRα replacing Lys557 of ERRγ. Strikingly, in the ERRα complex with compound 1a, Met517 is well ordered and is practically at the same position as Leu214 of PGC-1α bound.
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to ERRα (14). Lys457 of ERRγ cannot make similar hydrophobic interactions in the coactivator groove, so this substitution is a possible explanation for the differences in H12 positions.

In addition to Leu536 and Leu540 of the LXXLL motif, ERRα also utilizes Leu544 of H12 for interactions in the coactivator cleft. According to the sequence alignment, these residues would correspond to Met506, Phe510, and Leu514 of ERRα, but interestingly they are not pointing into the groove. Instead, Leu509, Met513, and Met517 of ERRα take up this role, enabled by a lengthening (compared with ERRα) of the H11/H12 loop structure by three residues. Knowledge of these unexpected interactions can further help in the design of peptide or non-peptide antagonists, which bind in the coactivator groove (instead of the LBP), but do not necessarily contain an LXXLL motif. Recently, ERRα-selective peptide antagonists containing an LXXLL motif were described, which were identified by screening combinatorial phage libraries (37).

CONCLUSION

In this study, we report the first x-ray structure of ERRα LBD in complex with an inverse agonist, cyclohexylmethyl-(1-p-tolyl-1H-indol-3-ylmethyl)-amine (compound 1a), to a resolution of 2.3 Å. Our data reveal for ERRα the multiple conformational changes in the LBP induced by the binding of compound 1a. These changes are the trigger for a novel mechanism of H12 displacement. In addition, they show an unexpected movement of H3 leading to an enlargement of the LBP and a surprising mode of H12 interaction in the coactivator binding groove. The structure also provides the basis for rational drug design to obtain isotype-specific inverse agonists of this potential new drug target. The finding that, despite a practically filled LBP in apoERRα, a suitable ligand can induce an opening of the cavity has broad implications for other orphan NRs. In particular, it is of interest for the recently published LBD crystal structures in the NGFI-B family (38–40), for which the LBP is completely open for ERRα, it is tempting to speculate that synthetic ligands (probably acting as inverse agonists) might also be found for members of the NGFI-B subfamily.

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REFERENCES

1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 835–839
2. Giguere, V. (1999) Endocr. Rev. 20, 689–725
3. Giguere, V., Yang, N., Segui, P., and Evans, R. M. (1988) Nature 331, 91–94
4. Vanacker, J. M., Pettersson, K., Gustafsson, J.-A., and Laudet, V. (1999) Nature 391, 91–94
5. Giguere, V. (2002) Trends Endocrinol. Metab. 13, 229–225
6. Luo, J., Sladek, R., Carrier, J., Bader, J.-A., Richard, D., and Giguere, V. (2003) Mol. Cell. Biol. 23, 7947–7956
7. Schreiber, S. N., Emter, R., Hock, M. B., Knutti, D., Cardenas, I., Podvines, M., Oakeley, E. J., and Kralli, A. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 6472–6477
8. Mootha, V. K., Handschin, C., Arlow, D., Xie, X., St Pierre, J., Shag, S., Yang, W., Altshuler, D., Puigserver, P., Patterson, N., Willy, P. I., Schulman, I. G., Heyman, R. A., Lander, E. S., and Spiegelman, B. M. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 6570–6575
9. Ariazi, E. A., Clark, G. M., and Mertz, J. E. (2002) Cancer Res. 62, 6510–6518
10. Ariazi, E. A., and Jordan, V. C. (2006) Curr. Top. Med. Chem. 6, 103–215
11. Bonneye, E., and Aubin, J. E. (2005) J. Clin. Endocrinol. Metab. 90, 3115–3121
12. Stein, B., Way, J. M., McDonnell, D. P., and Zuercher, W. J. (2006) Drugs of the Future 31, 427–436
13. Chen, S., Zhou, D., Yang, C., and Sherman, M. (2001) J. Biol. Chem. 276, 28465–28470
14. Kalten, J., Schlaeppi, J. M., Bitsch, F., Filippuzi, I., Schibl, A., Riou, V., Graham, A., Strauss, A., Geiser, M., and Fournier, B. (2004) J. Biol. Chem. 279, 49330–49337
15. Horard, B., and Vanacker, J. M. (2003) J. Mol. Endocrinol. 31, 349–357
16. Willy, P. J., Murray, I. R., Qian, J., Busch, B. B., Stevens, W. C., Martin, R., Mohan, R., Zhou, S., Ordentlich, P., Wei, P., Sapp, D. W., Horlick, R. A., Heyman, R. A., and Schulman, I. G. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 8912–8917
17. Busch, B. B., Stevens, W. C., Jr., Martin, R., Ordentlich, P., Zhou, S., Sapp, D. W., Horlick, R. A., and Mohan, R. (2004) J. Med. Chem. 47, 5939–5956
18. Greschik, H., Flaig, R., Renaud, J. P., and Moras, D. (2004) J. Biol. Chem. 279, 33639–33646
19. Chao, Y. H., Collins, J. L., Gaillard, S., Miller, A. B., Wang, L., Orband-Miller, L. A., Nolte, R. T., McDonnell, D. P., Willson, T. M., and Zuercher, W. J. (2006) Bioorg. Med. Chem. Lett. 16, 821–824
20. Wang, L., Zuercher, W. J., Consler, T. G., Lambert, M. J., Miller, A. B., Orband-Miller, L. A., McKee, D. D., Willson, T. M., and Nolte, R. T. (2006) J. Biol. Chem. 281, 37773–37781
21. Greschik, H., Wurtz, J. M., Sanglier, S., Bourguet, W., van Dorsellea, A., Moras, D., and Renaud, J. P. (2002) Mol. Cell 9, 303–313
22. Brzozowski, M. A., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O., Ohman, L., Greene, G. L., and Gustafsson, J. A. (1997) Nature 389, 753–758
23. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Agard, D. A., and Greene, G. L. (1998) Cell 95, 927–937
24. Pike, A. C., Brzozowski, M. A., Walton, J., Thorsell, A. G., Lai, Y. L., Gustafsson, J. A., and Carlquist, M. (2001) Structure (Cambridge) 9, 145–153
25. Strauss, A., Bitsch, F., Cutting, B., Fendrich, G., Graff, P., Liebetanz, J., Zurini, M., and Jahneke, W. (2003) J. Biomed. NMR 26, 367–372
26. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
27. Vagin, A., and Teplyakov, A. (1997) J. Appl. Crystallogr. 30, 1022–1025
28. Collaborative Computational Project, No4. (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
29. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. Sect. D Biol. Crystallogr. 53, 240–255
30. Lamzin, V. S., Perrakis, A., and Wilson, K. S. (2001) in International Tables for Crystallography (Rossmann, M. G., and Arnold, E., eds) Vol. F, pp. 720–722, Kluwer Academic Publishers Group, Dordrecht, Netherlands
31. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
32. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–299
33. Widmer, H., and Jahneke, W. (2004) Cell. Mol. Life Sci. 61, 580–599
34. Jahneke, W. (2002) ChemBioChem. 3, 167–173
35. Renaud, I., Bischof, S. F., Buhl, T., Floersheim, P., Fournier, B., Geiser, M., Halleux, C., Kalten, J., Keller, H., and Ramage, P. (2005) J. Med. Chem. 48, 364–379
36. Shiau, A. K., Barstad, D., Radek, J. T., Meyers, M. J., Nettles, K. W., Katzenellenbogen, B. S., Katzenellenbogen, J. A., Agard, D. A., and Greene, G. L. (2002) Nat. Struct. Biol. 9, 359–364
37. Gaillard, S., Dwyer, M. A., and McDonnell, D. P. (2007) Mol. Endocrinol. 21, 62–76
38. Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., Xu, H., Walker, N. P., and Perlmann, T. (2003) Nature 423, 555–560
39. Baker, K. D., Shewchuk, L. M., Kozlova, T., Makishima, M., Hassell, A., Wisely, B., Caravella, J. A., Lambert, M. H., Reinking, J. L., Krause, H., Thummel, C. S., Willson, T. M., and Mangelsdorf, D. J. (2003) Cell 113, 731–742
40. Flaig, R., Greschik, H., Peluso-Iltis, C., and Moras, D. (2005) J. Biol. Chem. 280, 19250–19258
41. DeLano, W. L. (2002) PyMOL, DeLano Scientific, San Carlos, CA