Structural Basis for the Deactivation of the Estrogen-Related Receptor \( \gamma \) by Diethylstilbestrol or 4-Hydroxytamoxifen and Determinants of Selectivity

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running title: ERR\( \gamma \) LBD bound to diethylstilbestrol or 4-hydroxytamoxifen
SUMMARY

The estrogen-related receptor (ERR) $\gamma$ behaves as a constitutive activator of transcription. While no natural ligand is known, ERR$\gamma$ is deactivated by the estrogen receptor (ER) agonist diethylstilbestrol and the selective ER modulator 4-hydroxytamoxifen, but does not significantly respond to estradiol or raloxifene. Here we report the crystal structures of the ERR$\gamma$ ligand-binding domain (LBD) complexed with diethylstilbestrol or 4-hydroxytamoxifen. Antagonist binding to ERR$\gamma$ results in a rotation of the side chain of F435 that partially fills the cavity of the apo-LBD. The new rotamer of F435 displaces the 'activation helix' (helix 12) from the agonist position observed in the absence of ligand. In contrast to the complexes of the ER$\alpha$ LBD with 4-hydroxytamoxifen or raloxifene, helix 12 of antagonist-bound ERR$\gamma$ does not occupy the coactivator groove, but appears to be completely dissociated from the LBD body. Comparison of the ligand-bound LBDs of ERR$\gamma$ and ER$\alpha$ reveals small but significant differences in the architecture of the ligand-binding pockets that result in a slightly shifted binding position of diethylstilbestrol and a small rotation of 4-hydroxytamoxifen in the cavity of ERR$\gamma$ relative to ER$\alpha$. Our results provide detailed molecular insight into the conformational changes occurring upon binding of synthetic antagonists to the constitutive orphan receptor ERR$\gamma$ and reveal structural differences with ERs that explain why ERR$\gamma$ does not bind estradiol or raloxifene and will help to design new selective antagonists.
INTRODUCTION

The estrogen-related receptors ERR\(\alpha\), ERR\(\beta\), and ERR\(\gamma\) (NR3B1, 2, 3) (1) form a subfamily of orphan nuclear receptors that share significant amino acid homology with the estrogen receptors ER\(\alpha\) and ER\(\beta\) [NR3A1, 2] (2, 3). Due to the high conservation in the DNA-binding domain (DBD)\(^5\), ERRs and ERs have overlapping DNA binding selectivity (4-6) and accordingly may co-regulate target genes in tissues in which they are co-expressed. ERR subfamily members have for example been shown to modulate the expression of ER target genes in bone (7, 8) or breast tissue (9, 10). Importantly, over-expression of ERR\(\alpha\) and ERR\(\gamma\) in samples from breast cancer patients correlates with unfavourable and favourable biomarkers, respectively (11). Therefore, these receptors might serve as prognostic markers themselves or even be targets for endocrine therapy in human breast cancer.

Despite their significant homology with ERs in the ligand-binding domain (LBD), ERRs do not (or only very weakly) respond to estradiol (E2) (2, 12). Furthermore, whereas ERs are ligand-activated receptors, ERRs are constitutively active (13-16), and a structural study confirmed that the ERR\(\gamma\) LBD can adopt a transcriptionally active conformation and interact with the steroid receptor coactivator 1 (SRC-1) in the absence of any ligand (12). Together, these observations suggest that ERRs are ligand-independent activators of transcription, whose activation potential may rather be determined by the presence of transcriptional coactivators (17-20).

While natural ERR agonists may not exist, putative endogenous ligands were predicted to act as antagonists due to the specific architecture of the ligand-binding pocket (LBP) observed for the ERR\(\gamma\) apo-LBD (12). Although no natural ligand is known to date, the ER agonist diethylstilbestrol (DES) and the selective ER modulator (SERM) 4-hydroxytamoxifen (4-OHT) have been identified as ERR antagonists (21-23). DES deactivates all three isotypes, while 4-OHT binds only to ERR\(\beta\) and ERR\(\gamma\). In contrast, the
SERM raloxifene (RAL) does not bind to ERRs (23) (summarized in Table 1). The activity of ERRα is also antagonized by the organochlorine pesticides chlordane and toxaphene (24), but binding of these substances to ERRα remains unclear (21). In contradiction to structure-based predictions (12), a recent study identified flavone and isoflavone phytoestrogens as ERR agonists (25), although binding of these substances to the receptors has not been demonstrated.

Nuclear receptor LBDs adopt a canonical antiparallel α-helical sandwich fold generally composed of 12 α-helices (H1 to H12) and a small β-sheet (26, 27). Agonist ligands trigger the LBD activation function (termed AF2) by stabilizing a defined conformation, in which H12 packs against the LBD body and together with H3 and H4 generates a hydrophobic coactivator binding surface. Coactivators typically bind via LXXLL sequence motifs (L denotes leucine and X any amino acid) that form amphipatic α-helices. Antagonist ligands interfere with the formation of an active LBD conformation and coactivator recruitment. In most documented cases AF2 antagonism is based on sterical interference between a bulky ligand extension protruding from the LBP and H12 in the agonist position, as exemplified by the ERα LBD bound to RAL or 4-OHT (28, 29) or the ERβ LBD complexed with ICI 164,384 (ICI) (30). Alternatively, 'small' antagonists without bulky extension can destabilize the active LBD conformation by promoting non-productive interactions between H12 and the LBD body, as exemplified by the action of 5,11-cis-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (THC; R,R enantiomer) on ERβ (31).

In crystallographic and recent fluorescence studies it has been attempted to more precisely correlate the conformation of the antagonist-bound ER LBD with the distinct biological activities elicited by SERMs (RAL, 4-OHT) or full ER antagonists (ICI compounds) in vivo (28-30, 32). In the crystallized complexes of the ERα LBD with RAL or 4-OHT a partial unfolding of the C-terminal end of H11 and structural adaptations of the
H11/H12 loop were observed, allowing H12 to bind to the coactivator groove (28, 29). In contrast, in the ERβ LBD/ICI complex H12 is not visible due to high mobility, since the terminal amide moiety of the ligand sterically precludes its packing against the coactivator groove (30). In turn, a high H12 mobility appears to result in an increased cellular ER turnover, accounting in part for the full antagonist activity of ICI compounds (30, 33). Fluorescence studies confirmed a partial unfolding of the C-terminus of H11 in ERα LBD/antagonist complexes in solution (32). Interestingly, full antagonists seem to have a lower H11 unfolding potential than SERMs, and subtle differences were observed between the spectroscopic signatures of RAL and 4-OHT.

In contrast to ERs, nothing is known about ligand-induced conformational changes of ERR LBDs. Here we report the crystal structures of the ERRγ LBD/DES complex (at 2.1 Å resolution) and of two ERRγ LBD/4-OHT complexes (at 2.2 Å and 3.2 Å resolution). Superimposition of the antagonist-bound structures with the ERRγ apo-LBD (refined to 2.4 Å resolution) in the transcriptionally active conformation establishes the conformational changes occurring upon DES or 4-OHT binding. Comparison of the ERRγ LBD/antagonist complexes with the ERα LBD bound to various ligands (E2, DES, 4-OHT, RAL) reveals differences in the architecture of the LBPs of ERα and ERRγ that do not permit efficient binding of E2 or RAL to ERRγ. Finally, our results suggest that (unlike observed in the complexes of the ERα LBD with RAL and 4-OHT) a highly mobile H12 interferes with the recruitment of coactivators to antagonist-bound ERRγ.
EXPERIMENTAL PROCEDURES

Protein production and purification

The hexahistidine-tagged mERRγ LBD (residues 229-458) was produced from a pET-15b expression vector (Novagen) in E. coli BL21(DE3) at 37 °C. After sonication of the bacterial pellet and ultracentrifugation the recombinant protein was first purified by cobalt affinity chromatography (TALON, Clontech). Affinity chromatography was carried out in batch at room temperature in buffer A [20 mM Tris-HCl (pH 8.0), 50 mM NaCl]. After three washes of the TALON resin with buffer A containing 5 mM imidazole (pH 8.0), the hexahistidine-tagged ERRγ LBD was eluted by increasing the imidazole concentration to 200 mM. The protein was further purified by gel filtration using a HiLoad 16/60 Superdex 200 column (Pharmacia) on a BioLogic workstation (BioRad). The LBD homodimer eluted in buffer A from the gel filtration column at the expected position as a single peak. The fractions containing purified protein (estimated purity > 95%) were pooled and concentrated to about 8 mg/ml.

Protein complex crystallization, data collection, and processing

Co-crystallisation with a 2-fold molar excess of DES or 4-OHT (Sigma) was carried out with the hanging-drop or the sitting drop vapour-diffusion method. [Hanging drop: 2 µl protein solution + 2 µl reservoir against 500 µl reservoir in 24-well VDX plates (Hampton Research). Sitting drop: 1 µl protein solution + 1 µl reservoir against 200 µl reservoir in 96-well CrystalQuick™ plates (Greiner).] For the ERRγ LBD/DES and one of the ERRγ LBD/4-OHT complexes, the PEG/Ion Screen (Hampton Research) allowed to find preliminary crystallization conditions. A second crystal form for the ERRγ LBD/4-OHT complex was found upon screening with the Crystal Screen II (Hampton Research). In the refined conditions, ERRγ LBD/DES crystals grew in sitting drops within a few days at 24 °C with a
reservoir containing 0.1 M Tris-HCl (pH 8.0), 0.1 M NaOAc, 18 % PEG3350 (cryo conditions: 0.1 M Tris-HCl (pH 8.0), 0.1 M NaOAc, 20 % PEG3350, 20 % ethylene glycol). ERRγ LBD/4-OHT crystals (crystal form 1) were obtained in hanging drops at 4 °C with a reservoir containing 0.1 M MES (pH 6.5), 1.6 M MgSO4 (cryo conditions: 0.1 M MES (pH 6.5), 1.6 M MgSO4, 13 % ethylene glycol). ERRγ LBD/4-OHT crystals of form 2 grew in hanging drops at 24 °C in reservoir containing 0.1 M Tris-HCl (pH 8.0), 0.1 M MgSO4, 14 % PEG3350 (cryo conditions: 0.1 M Tris-HCl (pH 8.0), 0.1 M MgSO4, 20 % PEG3350, 20 % ethylene glycol). New crystals of the ERRγ apo-LBD grew in the presence of a three-fold molar excess of a SRC-1 coactivator peptide (686-RHKILHRLLQEGSPS-700) (12) at 4 °C in hanging drops with a reservoir containing 100 mM Tris-HCl (pH 8.5), 1.6 M (NH4)2SO4, 20 % glycerol. (The mother liquor served as cryo-protectant.) X-ray diffraction data were collected at the ID14-1, the ID14-2, or the BM30 beam line at the ESRF in Grenoble, France. The data were integrated and scaled using the HKL2000 package (34).

**Structure determination, refinement, and comparison**

The crystal structures of the ERRγ LBD/DES and the ERRγ LBD/4-OHT complexes were solved by molecular replacement with AMoRe (35) using the C-terminally deleted ERRγ LBD homodimer (amino acids 235-440) (12) as search model. The structures were refined at the indicated resolution (Table 2) using CNS (36) and programs of the CCP4 package (37-40). Manual adjustments and rebuilding of the models were performed using the program 'O' (41). The final models were validated with PROCHECK (42). Data collection and structure refinement statistics are summarized in Table 2. For structure comparisons, the Cα traces of the models were superimposed from H3 (L265 in ERRγ; M343 in ERα) to the middle of H11 (F435 in ERRγ; L525 in ERα) using the lsq-commands of 'O' and default parameters. The
probe-occupied volume of the ERR\(\gamma\) LBP in the absence and presence of ligand was calculated with VOIDOO (43). Figures were generated with DINO (44).

RESULTS

Overall structure of the antagonist-bound ERR\(\gamma\) LBD

We solved the crystal structures of the ERR\(\gamma\) LBD in complex with DES (at 2.1 Å resolution, space group \(P_{2_1}\)) and with 4-OHT (crystal form 1 at 2.2 Å resolution, space group \(P_{4_1}2_2\); crystal form 2 at 3.2 Å resolution, space group \(P_{3_2}2_1\)) (Table 2). In addition, we further refined the structure of the ERR\(\gamma\) apo-LBD bound to a SRC-1 peptide (12) at 2.4 Å resolution (space group \(P_{4_1}2_12\)). In all cases, the ERR\(\gamma\) LBD crystallized in homodimeric form, thus ligand binding does not interfere with homodimer formation.

Comparison of the ERR\(\gamma\) apo-LBD with the DES and 4-OHT complexes revealed no major conformational changes for the main chain atoms from H1 to the middle of H11\(\&\). Some changes of the main chain include adaptations of the H1-H3 loop, which mainly result from crystal packing. In contrast, important structural perturbations of the main chain due to 4-OHT or DES binding affect the C-terminus of H11, the H11/H12 loop, and H12 (Table 3). First, in the ligand-bound complexes, the C-terminus of H11 is slightly bent away from the entrance of the LBP due to space requirements of the ligand. Second, in some subunits of the ERR\(\gamma\) LBD/4-OHT (but not the ERR\(\gamma\) LBD/DES) complexes present in the asymmetric unit (AU) the C-terminal end of H11 is unfolded. In contrast to ER\(\alpha\) LBD/SERM complexes (28, 29) partial H11 unfolding is not consistently observed in the ERR\(\gamma\) LBD/4-OHT complexes, but is only found in one subunit in both crystal forms. Finally, in all ligand-bound complexes the H12 region adopts distinct conformations or is found in different positions. The H11/H12 loop adapts to the constraints imposed by the exact position and conformation of H11 and H12 (Table 3).
In contrast to the complexes of the ERα LBD with RAL and 4-OHT (28, 29), in none of the ERRγ LBD/antagonist complexes does H12 pack against the coactivator groove of its respective LBD body. H12 is not visible most likely due to high mobility in the case of the ERRγ LBD/4-OHT complex (crystal form 2) and interacts with the coactivator groove of a neighbouring molecule in crystal form 1. For subunits 'A' and 'B' found in the AU of crystal form 1, this interaction requires different conformational adaptations of the H11/H12 loop and the N-terminus of the H12 region (Fig. 1A). In the ERRγ LBD/DES complex, the H12 region is not visible or unfolded in three out of four molecules (subunits 'A' to 'C'). H12 of the fourth molecule (subunit 'D') is stabilized through crystal contacts in a position that resembles the agonist conformation found in the ERRγ apo-LBD/SRC-1 complex (Fig. 1B). However, in this 'agonist-like' position, H12 of subunit 'D' is shifted by one residue along the α-helical axis with respect to the agonist H12 position in the ERRγ apo-LBD/SRC-1 complex. As a consequence the conformation of the H11/H12 loop is completely changed, and H12 occupies a position that in solution is most likely not stabilized by favourable contacts. Together, these observations suggest that in solution DES or 4-OHT binding strongly increases the mobility of H12, whose conformation and position in the crystals is apparently determined by packing interactions.

**Mechanism of DES- and 4-OHT-mediated antagonism**

Due to size limitations, DES or 4-OHT cannot bind inside the small ERRγ LBP (~ 220 Å³) observed in the apo-LBD (12). Consequently, DES binding induces structural adaptations that enlarge the volume of the ERRγ LBP to about 330 Å³. The 4-OHT binding niche differs from the DES cavity mainly around the amine moiety that extends onto the protein surface. The most prominent conformational change inside the LBP in response to DES and 4-OHT binding is a rotation of the side chain of F435 (H11), which mostly accounts for the increase...
in cavity volume (Fig. 2). The new rotamer of F435 would sterically clash with H12 in the agonist position, notably with the side chain of L454 in H12 (Fig. 2A). Thus, the DES- or 4-OHT-induced rotation of the side chain of F435 precludes formation of the canonical transcriptionally active conformation of the ERRγ LBD, which consequently impairs coactivator recruitment.

The binding niche of DES or 4-OHT is delineated by amino acid residues from H3 (L265, L268, C269, L271, A272, E275), H5 (W305, M306, L309, I310, V313, R316), the β-sheet (Y326), H7 (L342, L345, N346, I349), and H11 (A431, H434, F435) (Fig. 2 and &). The 'anti-estrogenic' amine moiety of 4-OHT forms additional contacts with W305 and F435&. Furthermore, the side chain of D273 (H3) on the ERRγ LBD surface reorients to form a hydrogen bond with the tertiary amine group of 4-OHT (Fig. 2B), which has also been observed with D351 in the ERα LBD/4-OHT complex (see Fig. 3B) (29). As in the corresponding ERα complexes (29), DES and 4-OHT are anchored on one side of the LBP by hydrogen bonds with E275 (H3) and R316 (H5), while DES forms another hydrogen bond with H434 (H11) on the other side of the cavity. Importantly, the side chain of E275, whose position is not well defined in the ERRγ apo-LBD due to high mobility, reorients towards the ligand and is tightly positioned in the DES and the 4-OHT complexes. In comparison, R316 and H434 only slightly reorient upon ligand binding. Other small structural changes inside the LBP relative to the apo-LBD concern the side chains of Y326 (β-sheet) and N346 (H7) interacting through a hydrogen bond, which adapt to space requirements of the ligand. Superimposition of the DES and 4-OHT complexes of the ERRγ LBD shows that neither the aromatic A-ring nor the A'-ring of DES superimpose exactly with the corresponding A- or B-ring of 4-OHT (Fig. 2B). This observation reveals a small degree of variability, by which different types of ligands can use the available space inside the ERRγ LBP.
Comparison with ERα and determinants of ligand binding selectivity

Structure superimposition reveals an overall similar mode of DES binding to ERRγ and ERα, the major principal difference between the complexes being a canonical agonist H12 position in the case of ERα, but various antagonist H12 conformations in the case of ERRγ (Fig. 3A). DES binding does not interfere with the agonist H12 position in ERα, because the side chain of L525 (corresponding to F435 in ERRγ) leaves enough space to accommodate the ligand without large conformational change. Interestingly, the binding position of DES is slightly shifted by about 0.5 Å in the ERRγ relative to the ERα cavity. This shift could result from differences in the relative positions of individual α-helices and thus the exact topology of the LBPs. Out of the secondary structure elements that contribute to the cavity, H3, H5, and the β-sheet superimpose well with only minor positional deviations. In contrast, significant shifts of 1.0 to 1.5 Å along the helix axis are observed for H7 and H11 of ERRγ (Fig. 3A and 3B). Both shifts restrict the available space in the ERRγ LBP, which may be the cause of the slightly altered DES binding position.

Importantly, compared with DES, the 4-OHT binding position differs more significantly in the LBPs of ERα and ERRγ (Fig. 3B). While the overall binding mode is conserved and the aromatic ligand A-ring and surrounding parts of the LBP superimpose relatively well in both complexes, the 4-OHT binding positions differ for the B-ring and the amine moiety. Sterical constraints imposed by L345 (H7) and F435 (H11) in ERRγ, (corresponding to I424 and L525 in ERα, respectively) lead to a small rotation of the 4-OHT molecule around C1 in the aromatic A-ring. A 4-OHT binding position in the LBP of ERRγ as observed in ERα would result in too close contacts with L345 (~ 3 Å) and F435 (~ 2.8 Å).

These differences in the architecture of the LBPs of ERRγ and ERα provide a good explanation why E2 and RAL do not bind to ERRγ with significant affinity. Superimposition
of ERα LBDs in complex with different ligands shows that E2 and RAL protrude deeper into the LBP towards I424 than 4-OHT (Fig. 3B and 3C) resulting in conformational adaptations including distinct side chain conformations of I424 (H7) (28, 29). In contrast, it appears that despite the dynamic behavior of proteins, the space restrictions in the cavity of ERRγ imposed by the shifted position of H7 cannot be compensated in an energetically favourable manner by a conformational change of the side chain of L345. Thus, the altered position of H7 and the presence of L345 in ERRγ (replacing I424 in ERα) seem to account mostly for the lack of E2 or RAL binding to this receptor.

**In one of the ERRγ LBD/4-OHT complexes the packing of H12 against the coactivator groove of a neighbouring molecule is determined by a fortuitously co-crystallized bile acid molecule**

Finally, we compared the H12/coactivator groove interactions in the 4-OHT complexes of ERRγ (crystal form 1) and ERα. In ERα H12 packs tightly against its respective LBD body and mimics coactivator binding by inserting L536 and L540 into the coactivator cleft (29). In contrast, in ERRγ the interaction of H12 with the LBD body of a neighbouring molecule is determined by the presence of a fortuitously co-crystallized molecule. During refinement the clearly contoured, additional electron density allowed us to identify this molecule as cholic acid (or a closely related bile acid) (Fig. 4A). The bile acid is bound close to the entrance of the LBP within the proximity of the protruding amine moiety of 4-OHT (shortest distance ~ 3.2 Å) and occupies together with the C-terminal portion of H12 the hydrophobic coactivator groove (Fig. 4B). Comparison of both ERRγ subunits present within the AU shows that most regions including H3, cholic acid, and the C-terminal part of H12 superimpose well, while the H11/H12 loop and the N-terminus of H12 adopt distinct folds due to different relative H12 positions (Fig. 1A and &). In the middle of H12, F450 of ERRγ interacts with the coactivator
cleft in a roughly similar manner as L540, the corresponding residue in ERα (Fig. 4B).
Packing of the N-terminal portion of the ERRγ H12 against the coactivator cleft of a
neighbouring molecule, however, is precluded by the presence of the cholic acid molecule and
determined by the distinct conformations of the H11/H12 loop in both ERRγ subunits.
Consequently, M446 of ERRγ does not form an interaction equivalent to that of the
corresponding L536 in ERα.

DISCUSSION

Mechanism of ERRγ deactivation

In this study we compare the crystal structures of the ERRγ LBD bound to the antagonists
DES or 4-OHT with the apo-LBD in the transcriptionally active conformation. Our
comparisons establish that DES- and 4-OHT-mediated antagonism is based on the rotation of
the side chain of F435, which upon ligand binding flips out of the LBP and sterically
interferes with H12 in the agonist position. As a consequence, H12 is dissociated from the
LBD body and coactivator binding is impaired. These ideas are in agreement with previous
results showing that mutation of F435 to leucine (the corresponding residue in ER) abolishes
the antagonist action of DES on ERRγ in functional assays (12). Since 4-OHT binding also
requires a rotation of the side chain of F435, the ligand amine moiety protruding from the
LBP is not primarily responsible for the antagonist action of 4-OHT on ERRγ.

Antagonist ligand effects on ERRγ and ERs

AF2 antagonists of ERs have been classified as 'active' if a bulky ligand extension interferes
with the agonist H12 position (RAL, 4-OHT, ICI), and as 'passive' in the case of small ligands
(THC) (28-31). Deactivation of ERRγ by DES (a ligand without bulky extension) provides a
particular case of 'active' antagonism, in which a small ligand acts as antagonist by inducing the rotation of the side chain of a single residues (F435) inside the LBP.

In an attempt to correlate antagonist-induced conformational changes of the ER LBD with ligand activities \textit{in vivo}, AF2 antagonists with bulky extension have been further subclassified according to their H11 'unfolding potential' and their potential to increase the mobility of H12 or to promote H12 packing against the coactivator cleft (28-30, 32). While most observations from structural and fluorescent studies appear consistent, questions remain about the dynamic behaviour of H12 in solution, since the packing of H12 against the coactivator cleft observed in the crystal structures of the ER\textsubscript{α} LBD with RAL and 4-OHT may be due to the crystallization conditions. In fact, to our knowledge the dynamic behaviour of H12 of ER in the presence of SERMs or full antagonists has never been comparatively analysed in fluorescence anisotropy experiments as previously applied for the wild-type or mutant PPAR\textsubscript{γ} LBD (45).

Similarly, the effects of ligand binding on the conformation of the ERR\textsubscript{γ} LBD and the dynamic behaviour of H12 have not been addressed in fluorescence studies. In the absence of such data, the presented crystal structures allow first insight into the conformational changes induced by DES and 4-OHT. Most parts of the ERR\textsubscript{γ} LBD (H1 to the middle of H11) do not undergo significant conformational changes upon antagonist binding. Structural adaptations affect the C-terminal part of H11, which slightly bends away from the entrance of the LBP due to space requirements of the ligand, but only unfolds in individual subunits within the AU of the ERR\textsubscript{γ} LBD/4-OHT complexes. Interestingly, DES and 4-OHT may have distinct 'unfolding potentials', since we do not observe partial H11 unfolding in the DES complexes. Binding of both antagonists, however, dissociates the H12 region from the LBD body. The H12 positions and conformations observed in the different complexes seem to be random and dictated by crystallisation conditions or crystal packing rather than correlate with ligand
characteristics. Notably, unlike observed for the ERα LBD/4-OHT complex (29), H12 does not pack against the coactivator cleft of its respective LBD body in the ERRγ LBD/4-OHT complexes. Since the length of the H11/H12 region is identical in both receptors, differences in the amino acid sequence may explain this observation. H12 of ERα contains an LXXLL 'coactivator' motif, of which L536 and L540 mediate the anchoring of H12 in the coactivator cleft in the RAL and the 4-OHT complex (28, 29). In contrast, no LXXLL motif is present in H12 of ERRγ, and the corresponding residues (M446 and F450, respectively) may not allow efficient interactions of H12 with the coactivator groove. Therefore, the dissociation of H12 from the LBD body upon DES or 4-OHT binding to ERRγ rather resembles the action of the fully antagonistic ICI compounds on ERs. However, the in vivo consequences of antagonist binding to ERRγ [e.g. an enhanced receptor degradation as for ER/ICI complexes (30, 33) or potential tissue-specific effects as for ERα/SERM complexes (46)] remain to be established in future studies.

A 'fortuitous' cholic acid molecule bound at the surface of the ERRγ LBD

Unexpectedly, in crystal form 1 of the ERRγ LBD/4-OHT complex, cholic acid (or a closely related bile acid) was co-crystallized. One possible explanation for the presence of cholic acid is that the molecule has been co-purified from the E. coli expression host, since bacteria present in the human colon are known to be involved in the metabolism of bile acids that escape the enterohepatic circulation (between the liver, gallbladder, and intestines) (47). Cholic acid together with parts of H12 from a neighbouring molecule covers the hydrophobic coactivator groove and thus stabilizes the crystal packing, a phenomenon that has been previously observed for a detergent molecule shielding the hydrophobic cleft in crystals of the RARγ LBD (48). The proximity of cholic acid to the entrance of the LBP and the amine moiety of 4-OHT raises the possibility that ligands may be designed that further extend onto
the ERRγ LBD surface. However, such an approach will require a more careful analysis of the 'binding' mode of cholic acid.

Currently, we do not see any physiological relevance for the observed association of cholic acid with the ERRγ LBD. Although ERRγ seems to be expressed at low levels in the small intestine and the liver (13, 49-51), the binding position of the bile acid at the ERRγ surface is clearly determined by the crystal packing and only compatible with an antagonist, but not an agonist LBD conformation. It therefore also remains unclear how cholic acid was 'fortuitously' co-purified with the ERRγ apo-LBD.

**Ligand binding selectivity of ERRs**

ERRs have been shown to bind DES (all ERRs) and 4-OHT (ERRβ and ERRγ), but not E2 or RAL (21-23). The comparison of the DES and 4-OHT complexes of the ERRγ and the ERα LBDs reveals small but significant differences in the architecture of the LBP that explain ligand binding selectivity. The slightly shifted binding position of DES and the small rotation of 4-OHT in the LBP of ERRγ apparently result from space restrictions due to the presence of L345 (I424 in ERα) and - relative to ERα - a shifted position of H7. Furthermore, the rotated side chain of F435 (L525 in ERα) 'pushes' the 4-OHT amine moiety towards D273. Importantly, in ERα I424 can adapt through conformational changes to ligands such as E2 or RAL that protrude much deeper into the cavity than 4-OHT (28, 29). In ERRγ, however, even taking into account protein dynamics, equivalent conformational adaptations of L345 apparently cannot occur in an energetically favourable manner, thus precluding E2 or RAL binding. These ideas are supported by previous results showing significant binding of E2 to the mutants ERRγ LBD(L345I) and ERRγ LBD(L345I/F435L) in non-denaturing mass spectrometry analysis (12). Together, our observations suggest that synthetic estrogens which as RAL deeply protrude into the ER LBP towards I424 are unlikely to bind to ERRγ (or other
ERR isotypes), whereas synthetic estrogens, which similar to 4-OHT do not completely fill the space around I424 are also potential ERR ligands.

In a recent study Suetsugi et al. (25) identified certain flavone and isoflavone phytoestrogens as ERR agonists, albeit without demonstrating their binding to ERR LBDs. The authors used a model of the ERRα LBD based on the agonist-bound ERα LBD to predict the presence of a LBP that can accommodate flavone or isoflavone ligands without interfering with the agonist conformation. Our superimposition of the antagonist-bound ERRγ LBDs with the ERβ LBD/genistein complex (52) indicates a possible sterical interference between the side chain of L345 of ERRγ and O4 (~2.2 Å), C3 (~3 Å), and C4 (~2.9 Å) of genistein. In comparison, small adaptations of the LBP might permit the binding of daidzein, which lacks the hydroxyl group on C4 of genistein. However, superimposition with the ERRγ apo-LBD clearly predicts daidzein or related molecules to act like DES and 4-OHT as antagonists. Since our models of the ERRα and the ERRβ LBD, which are based on the ERRγ rather than the more distantly related ERα LBD, suggest an overall very similar architecture of the LBPs, our prediction is valid for all ERR isotypes. Structure determination of the apo and ligand-bound LBD of ERRα and ERRβ will eventually clarify this issue.

Assuming that the overall architecture of the LBP is conserved among ERRs, the ERRγ LBD crystal structures provide a good starting point to attempt the design of isotype-selective antagonists. An important discriminative feature between the LBPs of ERRβ and ERRγ are Y326 (β-sheet) and N346 (H7) that form a hydrogen bond in ERRγ. In ERRβ these residues correspond to Y301 and Y321, respectively. The different size and hydrogen bonding properties of N346 (ERRγ) and Y321 (ERRβ) could be essential in the design of ERRβ- or ERRγ-selective ligands. In ERRα the corresponding residues are F382 and G402, respectively. Especially, the small residue G402 may allow to modify ligands such that the
additionally available space is filled, possibly resulting in higher affinity and ERRα selectivity.

Our ERRα LBD model suggests that the receptor does not bind 4-OHT due to the side chain of F328 (H3), corresponding to A247 in ERRβ and A272 in ERRγ (12). F328 (H3) and F495 (H11; corresponding to F435 in ERRγ) are predicted to almost completely fill the ERRα LBP. In agreement with this model, ERRα binds 4-OHT upon mutation of F328 to alanine (23), whereas an ERRγ (A272F) mutant no longer responds to 4-OHT (12). Since the ERRα cavity appears to be filled, dramatic conformational changes may occur upon the binding of DES. This idea is interesting with respect to the recently published LBD crystal structures of Nurr1 (53) and DHR38 (the Drosophila homologue of NGFI-B) (54), in which the LBP is completely filled with four aromatic residues conserved within the NGFI-B subfamily. The authors propose that these orphan receptors are unlikely to be ligand-regulated since no apparent conformational changes of the aromatic residues could open a cavity. However, given the dramatic conformational changes that may occur upon the binding of DES to ERRα and the high adaptability observed for the LBPs of PXR (55) and EcR (56), it is tempting to speculate that (synthetic) ligands might be identified for members of the NGFI-B subfamily.

In summary, the results presented in this study establish the conformational changes of the ERRγ LBD occurring upon DES or 4-OHT binding and reveal determinants of selective ligand binding and differences in the dynamic behaviour of H12 of ERRs vs. ERs.

Acknowledgements

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We are grateful to Joanne McCarthy, Steffi Arzt, Stéphanie Monaco, Carlo Petosa, and Philippe Carpentier, our local contacts at the ESRF (Grenoble).

REFERENCES


FOOTNOTES

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§ The abbreviations used are: AF, activation function; AU, asymmetric unit; DBD, DNA-binding domain; DES, diethylstilbestrol; E2, estradiol; ER, estrogen receptor; ERR, estrogen-related receptor; H, α-helix; 4-OHT, 4-hydroxytamoxifen; ICI, ICI 164,384; LBD, ligand-binding domain; LBP, ligand-binding pocket; RAL, raloxifene; r.m.s.d., root mean square deviation; SERM, selective estrogen receptor modulator; SRC-1, steroid receptor coactivator-1; THC, 5,11-cis-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol.

§ The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org) [ERRγ apo-LBD: 1KV6, ERRγ LBD/DES: 1S9P, ERRγ LBD/4-OHT (crystal form 1): 1S9Q, and ERRγ LBD/4-OHT (crystal form 2): 1VJB].

& data not shown

FIGURE LEGENDS

FIG. 1: H12 positions found in complexes of the ERRγ LBD with 4-OHT or DES.

(A) Superimposition of subunits 'A' (orange) and 'B' (black) present in the AU of the ERRγ LBD/4-OHT complex (crystal form 1). In both cases, H12 packs against the coactivator groove of a neighbouring molecule with distinct adaptations of the H11/H12 region. Amino acids 443 and 444 of subunit 'A' and 439 to 444 of subunit 'B' are not well defined by electron density and have been omitted from the final model. (B) Shifted agonist-like position of H12 in subunit 'D' of the ERRγ LBD/DES complex (green). The corresponding parts of the ERRγ
apo-LBD (yellow) with H12 in the transcriptionally active position have been superimposed. In the DES complex the conformation of the H11/H12 loop and the position of H12 are determined by crystal packing and are most likely of no physiological relevance.

**FIG. 2: Conformational changes inside the ERRγ LBP upon antagonist binding.**

(A) Superimposition of selected parts of the ERRγ LBD/DES complex (green) with the ERRγ apo-LBD (yellow). For the apo-LBD, H12 in the agonist position has been included. The largest side chain movements in response to DES binding are observed for F435 and E275, while Y326, N346, and H434 adapt to a lesser extent. The rotation of the side chain of F435 upon DES binding results in sterical interference with H12 in the agonist position. (B) Superimposition of selected parts of the ERRγ LBD complexed with 4-OHT (orange) or DES (green). The ligands occupy slightly different positions inside the ERRγ cavity. (C) DES (left) or 4-OHT (right) bound to the ERRγ LBD fitted into 2Fo-Fc electron density maps.

**FIG. 3: Comparison of the LBPs of ERRγ and ERα.**

(A) Superimposition of selected parts of the ERRγ LBD/DES complex (green) with the ERα LBD/DES complex (red) [PDB: 3ERD]. While the overall ligand binding mode is very similar in both complexes, the DES binding position is shifted by about 0.5 Å in the cavity of ERRγ relative to ERα. In contrast to ERRγ, H12 of the ERα LBD can occupy the agonist position since there is no steric clash with L525 (corresponding to F435 in ERRγ). (B) Superimposition of selected parts of the ERRγ LBD/4-OHT complex (orange) with the ERα LBD/4-OHT complex (blue) [PDB: 3ERT]. In ERRγ, the 4-OHT binding position is slightly rotated and mainly differs around the B-ring and the amine moiety. The altered 4-OHT binding results from sterical constraints imposed by L345 (I424 in ERα), a slightly shifted relative position of H7, and the side chain of F435 (L525 in ERα). (C) Left: superimposition
of 4-OHT (blue), E2 (light grey), and RAL (black) as observed in the LBP of the respective ERα complexes [PDB ERα/E2: 1ERE, ERα/RAL: 1ERR]. Right: superimposition of 4-OHT as observed in the cavity of ERRγ (orange) with E2 (light grey), and RAL (brown) bound to ERα. E2 and RAL protrude deeper into the cavity of ERα than 4-OHT and do not bind to ERRγ due to insufficient space in the LBP.

**FIG. 4: Interactions of H12 with the coactivator groove in the ERα LBD/4-OHT and the ERRγ LBD/4-OHT complex (crystal form 1).**

(A) Fitting of the fortuitously co-crystallized cholic acid molecule (light grey) into a 2Fo-Fc electron density map. (B) Superimposition of selected parts of the ERRγ LBD (orange) and the ERα LBD (blue) bound to 4-OHT. In the ERα LBD/4-OHT complex, H12 packs against the coactivator cleft of the respective LBD body with L536 and L540 mimicking LXXLL coactivator interactions. In the ERRγ LBD/4-OHT complex (subunit 'B' in crystal form 1), H12 interacts with the coactivator groove of a neighbouring molecule. Binding is distinct from that in ERα due to the absence of a LXXLL motif in H12 of ERRγ. The alternative packing interactions between H12 and the coactivator groove are rather determined by the fortuitously co-crystallized cholic acid molecule that occupies part of the cleft.
### Table 1: Effect of selected ligands on the AF2 of ERs and ERRs

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A dash indicates that the respective ligand does not or only very weakly affect the transcriptional activity of the nuclear receptor.
### Table 2: Data collection and refinement statistics

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\(^a\) Values in parentheses are for the highest resolution shell.

\(^b\) R\(_{\text{sym}}\)(I) = \(\Sigma_{hkl} \Sigma_i I_i(hkl) - \langle I(hkl)\rangle / \Sigma_{hkl} \Sigma_i I_i(hkl)\), where \(\langle I(hkl)\rangle\) is the average intensity of the multiple \(I_i(hkl)\) observations for symmetry-related reflections.

\(^c\) R\(_{\text{cryst}}\) = \(\Sigma_{hkl} ||F_{\text{obs}}|| - k|F_{\text{calc}}||/\Sigma_{hkl} ||F_{\text{obs}}||\), where the test set (T = 5 % of reflections) is omitted in the refinement.

\(^d\) R\(_{\text{free}}\) = \(\Sigma_{hkl\in T} ||F_{\text{obs}}|| - k|F_{\text{calc}}||/\Sigma_{hkl\in T} ||F_{\text{obs}}||\), where the test set (T = 5 % of reflections) is omitted in the refinement.
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</table>
FIG. 2C

DES (ERRγ)  4-OHT (ERRγ)
FIG. 3A
FIG. 3C

4-OHT (ERα)  4-OHT (ERRγ)
E2 (ERα)      RAL (ERα)
cholic acid (ERRγ)

FIG. 4A
Structural basis for the deactivation of the estrogen-related receptor gamma by diethylstilbestrol or 4-hydroxytamoxifen and determinants of selectivity
Holger Greschik, Ralf Flaig, Jean-Paul Renaud and Dino Moras

J. Biol. Chem.  published online May 24, 2004

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