Crystal Structure of a Mutant hERα Ligand-binding Domain Reveals Key Structural Features for the Mechanism of Partial Agonism*

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The crystal structure of a triple cysteine to serine mutant ERα ligand-binding domain (LBD), complexed with estradiol, shows that despite the presence of a tightly bound agonist ligand, the protein exhibits an antagonist-like conformation, similar to that observed in raloxifen and 4-hydroxytamoxifen-bound structures. This mutated receptor binds estradiol with wild type affinity and displays transcriptional activity upon estradiol stimulation, but with limited potency (about 50%). This partial activity is efficiently repressed in antagonist competition assays. The comparison with available LBD structures reveals key features governing the positioning of helix H12 and highlights the importance of cysteine residues in promoting an active conformation. Furthermore the present study reveals a hydrogen bond network connecting ligand binding to protein transactivation. These observations support a dynamic view of H12 positioning, where the control of the equilibrium between two stable locations determines the partial agonist character of a given ligand.

Steroid hormones regulate the transcription of target genes in the cell by binding to transcription regulators that belong to the superfamily of nuclear receptors. All members of this family display a modular structure composed of six domains (A–F). The E region constitutes the ligand-binding domain (LBD)1 containing a ligand-dependent transactivation function (AF-2) (1, 2). The transcriptional activity of nuclear receptors is mediated by interactions with the transcriptional machinery through various corepressors and coactivators (3). Their ability to modulate gene expression in a ligand-regulated manner is based on the position of helix H12 carrying the AF2-AD transactivation function (4). Several positions of H12 have been observed (5). In the absence of ligand, H12 has been shown to be exposed to solvent (6). Ligand binding triggers a conformational change that results in the repositioning of H12 on the core of the LBD, closing the ligand binding pocket like a lid (7). This is referred to as the mouse trap mechanism (8). In agonist-bound LBDs a surface suitable for coactivator binding is then created (9–12). In most agonist-bound complexes (11, 12), H12 has been observed positioned in a structurally conserved cleft where the LXXLL motif of the coactivator molecule binds. These observations suggest a mechanism for antagonism where H12 and the coactivator compete for a common binding site. Note that the agonist position of H12 is unique, whereas its position in antagonist-bound complexes is not. Therefore knowledge of the features responsible for inducing and stabilizing a given conformation is a key step in understanding the initial events of nuclear receptor transactivation.

Several crystal structures of both ER isotypes (ERα and ERβ) have been solved in complex with natural and synthetic ligands (12–16). The natural ligand 17β-estadiol acts as a pure agonist on both isotypes. Others typified by EM-800 and ICI164,384 are described as pure antagonists (17). A third category of ligands displaying cell-type and promoter dependence in ER regulation are referred to as selective ER modulators (SERMs) (18). SERMs such as raloxifen and 4-hydroxytamoxifen efficiently antagonize the AF2, but not the AF1 function, and act as a pure antagonist (19) in ERβ, which seems to lack a functional AF1 domain (20). The features responsible for inducing a given conformation and stabilizing it are crucial to the definition of the optimal stereoechemical and biophysical specificity of a ligand. Here we present the comparison of the wild type hERα LBD crystal structure (16) with that of a mutant protein complexed with estradiol, where three cysteine residues were mutated in serine. The mutant protein binds estradiol with wild type affinity but has limited transcriptional capacity. In the structure of the Cys→Ser triple mutant hERα LBD, we observed an antagonist conformation despite the presence of a tightly bound estradiol in the ligand-binding cavity. This antagonist conformation, together with the transcriptional activity of the single, double, and triple cysteine to serine mutant receptors, supports the view of the agonist-antagonist equilibrium of H12 and gives some insight into the molecular mechanism for the conformational switch that drives the receptor in an agonist or antagonist conformation.

EXPERIMENTAL PROCEDURES

Protein Production, Purification, and Crystallization—The Cys→Ser triple mutant hERα LBD (Lys274→Pro374), in fusion with six histidine residues is produced using the pET15b(Escherichia coli BL21(DE3) expression system and purified by a zinc affinity column, ion exchange, and gel filtration. The purification procedure is similar to that of the wild type ER LBD (16). Crystals were obtained by vapor diffusion at 4 and 17 °C using hanging drops made by mixing 1 μl of protein solution (2.5 mg/ml) with 1 μl of reservoir solution (12% poly-
Table I

Data processing, phase determination and refinement statistics of Cys → Ser triple mutant structure (P622, a = b = 58.6 Å, c = 276.0 Å)

| Resolution (Å) | Native | P1 | P2 | Au |
|----------------|--------|----|----|----|
|                | 2.0    | 2.9| 3.4| 2.9|
| Unique reflections | 20231 | 6917 | 4415 | 6793 |
| Completeness (%) | 99.9 | 98.3 | 97.0 | 96.8 |
| Multiplicity | 10.0 | 5.0 | 3.0 | 4.0 |
| Rsym (%) (last shell) | 5.1 (33) | 6.9 | 6.2 | 6.5 |
| Number of sites | 81 | 81 | 81 | 81 |
| Rfinal (centric/acentric/anomalous) | 0.64/0.64/0.91 | 0.65/0.59/0.86 | 0.79/0.83/0.94 |
| Phasing power (centric/acentric/anomalous) | 1.92/5.14 | 2.12/9.22/2.12 | 1.41/6.12 |
| Rfree (%) | 15–2.2 | 15% | 15% | 15% |
| Working set | 14756 | 14756 | 14756 | 14756 |
| Test set | 715 | 715 | 715 | 715 |
| Number of water molecules | 466 | 466 | 466 | 466 |
| Working R factor (%) | 22.3 | 22.3 | 22.3 | 22.3 |
| Free R factor (%) | 27.3 | 27.3 | 27.3 | 27.3 |
| Rmsd bond lengths (Å) | 0.0081 | 0.0081 | 0.0081 | 0.0081 |
| Rmsd bond angles (degree) | 1.172 | 1.172 | 1.172 | 1.172 |
| Average B-factor | 34.3 | 34.3 | 34.3 | 34.3 |

Crystal Structure of a Mutant hERα LBD

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| Rfree (%) | 15–2.2 | 15% | 15% | 15% |
| Working set | 14756 | 14756 | 14756 | 14756 |
| Test set | 715 | 715 | 715 | 715 |
| Number of water molecules | 466 | 466 | 466 | 466 |
| Working R factor (%) | 22.3 | 22.3 | 22.3 | 22.3 |
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| Rmsd bond lengths (Å) | 0.0081 | 0.0081 | 0.0081 | 0.0081 |
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| Average B-factor | 34.3 | 34.3 | 34.3 | 34.3 |
using the Bradford technique. The binding assays were all performed in the presence of a total protein concentration of 5 mg/ml to avoid retention of the receptor in complex with the ligand, thus the crude extract was diluted with soluble proteins from untransformed bacteria. Receptor quantification was achieved in the presence of 10−6 M [6,7-3H]estradiol (β. I. du Pont de Nemours & Co.E. L.) with (for nonspecific binding) or without (for total binding) an excess of cold estradiol (2.10−8 M) and increasing amounts of crude extract. After 5 h at 4°C, bound (B) and free (F) ligands were separated by dextran-coated charcoal (4% Norit A charcoal, 0.4% dextran T-70 in the binding buffer). This mixture was left on ice for 5 min and centrifuged at 12,000 × g for 5 min. The supernatant was removed for scintillation counting (30). Specific binding was plotted against the volume of crude extract for receptor quantification. For the Kd determination the crude extract was incubated with increasing concentrations (from 10−10 to 10−7 M) of radiolabeled estradiol at 4°C overnight. Each measure was done in triplicate for Scatchard analysis. The variation of B/F as a function of B was analyzed as described previously (31).

RESULTS

The Molecular Structure—The structure of the triple mutant ERα LBD (Fig. 1b) exhibits the predominantly ß-helical fold observed for all nuclear receptors. The superposition over the wild type structure in complex with estradiol (16) (Fig. 1a) leads to an r.m.s. deviation of 0.54 Å over 211 Ca atoms (H1, H3-H8, H9-H11). The most striking conformational difference between these two structures is the different positioning of helix H12.

The Ligand Binding Pocket—The overall structure of the pocket is similar in the wild type and the Cys → Ser triple mutant. All side chains of the hydrophobic residues lining the pocket are at the same position. This explains the fact that the dissociation constants at equilibrium (Kd) between the wild type and the Cys → Ser triple mutant for estradiol are very close (Table II and Fig. 2). The main differences are found on the 17-OH and 3-OH side of estradiol (Fig. 3). Due to the antagonist position of H12, the cavity in the triple mutant is not sealed as in the wild type structure. On the O17 side (ß-ring side) of estradiol, the cavity reaches the surface of the protein and results in a much larger volume than the wild type ligand binding pocket. This channel is partially filled with water molecules, forming numerous hydrogen bonds with the protein. On the 3-OH side (A-ring side) an open narrow tunnel filled with water molecules is present in the mutant. In the wild type, this tunnel is almost closed and only the water molecule interacting with the 3-OH of estradiol is present. In the Cys → Ser triple mutant the estradiol A-ring superimposes perfectly with its equivalent group in the wild type complex, whereas the ß-ring is slightly shifted, as shown by the displacement of the C17, which moves 0.5 Å closer to helices H3 and H12 (Fig. 3).

The superposition of the Cys → Ser triple mutant with the hERα-raloxifen complex reveals that Asp351, which anchors the ammonium moiety of raloxifen, adopts the same conformation in both structures. All the helices, including H12, match perfectly (r.m.s. deviation: 0.5 Å), the protruding chain of raloxifen fitting perfectly in the water channel observed in the mutant structure. Furthermore, in this structure, electron density could be observed for the loop 11–12, a region that was not seen in the ER LBD/raloxifen structure. This loop includes the C terminus of the shortened helix H11, in particular Lys528, which points toward the ligand binding pocket channel.

ER pure antagonists exhibit acidic moieties in their protruding chain and are thus unlikely to interact with Asp351 as do raloxifen and tamoxifen. The present structure suggests Lys528 as a potential hydrogen bond partner for pure antagonist ligands bearing sulfanyl-like (IC182780) (32) or sulfonyl-like (RU58688) (33) groups in their protruding chain. Such a contact would cross the AF2 AD groove and hamper the agonist positioning of H12.

Cumulative Effect of Cys → Ser Mutations on ER Transactivation Potency—Interestingly each single Cys → Ser mutation...
(C381S, C417S, C530S) contributes equally to the observed transactivation reduction for the triple mutant receptor (Fig. 4 and Table II). Each single Cys → Ser mutation decreases the full-length receptor's activity by about 20%, whereas double mutations reduce CAT activity by ~40%, and the triple mutant exhibits a 56% decrease. Maximal wild type activity could not be restored even in the presence of saturating estradiol concentrations. Moreover each time a cysteine is mutated to serine the contribution of the ligand-independent transactivation function AF1 is removed. The Cys→Ser triple mutant receptor (GAL-ER) wild type and triple mutant on concentrations. Moreover each time a cysteine is mutated to serine the contribution of the ligand-independent transactivation function AF1 is removed. The Cys→Ser triple mutant displays 48% activity compared with the wild type GAL-ER, which is very close to the value observed for the full-length triple mutant receptor (44%, Fig. 2b). These data showed that the residual transactivation activity in the triple mutant is not due to AF1. Antagonist competition assays with raloxifen revealed that this SERM represses more efficiently the estradiol-dominating groove. In the present structure this residue, which is now serine, is still solvent-accessible and is involved in a water-mediated hydrogen bond network lining the helix H12. This residue is accessible in the mutant structure where H12 is in the antagonist position. In the wild type structure the cysteine residue is precluded from the solvent by helix H12. In the present structure this residue, which is now serine, is still solvent-accessible and is involved in a water-mediated hydrogen bond network lining the helix H12 agonist binding groove. In the mutant receptor, a positioning of H12 in the agonist groove is possible but would require the shortening of helix H3 by one turn at its N terminus. The conformational reorganization includes the last 10 residues of loop 1–3. Interestingly, the tamoxifen complex is nearly identical to the triple mutant in this region, whereas the raloxifen-bound structure exhibits a wild type conformation without shortening. Note that the overall Cys→Ser triple mutant structure is closer to that of the tamoxifen-bound LBD than to the raloxifen one. Nevertheless some differences remain between the mutant and tamoxifen structures, especially in the loop 6–7 region, which is shifted by more than 3.0 Å (Glu419 and Gly420) toward the core of the protein. This large movement in the antagonist structure is most likely induced by tamoxifen, whose aromatic ring is almost perpendicular to the estradiol d-ring and superimposes on the position 17. This movement of loop 6–7, filling the cavity, encroaches on the N terminus of helix H3, which thus adopts a conformation similar to that of the mutant protein.

The Conformational Change Induced by a Hydrogen-bonding Network Observed in Agonist Complexes—The shortening of helices H3 and H11 affects the surrounding structure, in particular a hydrogen bond network present in the wild type involving the conserved Glu419 on the loop H6-H7, Lys531 on the end of H11, and Glu339 at the N-terminal part of helix H3 (Fig. 5c). The disruption of these interactions is a direct consequence of both C417S and C530S mutations. The triple mutant E339A,E419A,K531A, which is unable to form the hydrogen-bonding network, further underlines the importance of this network for H12 positioning. We have analyzed the transcriptional capacity of this Ala mutant and compared it to the Cys→Ser mutations using GAL-ER constructs (Fig. 2b). The Ala triple mutant displayed 62% activity compared with the wild type receptor, which is comparable with the value of 64% observed with the C417S,C530S double mutant. The similarity of effects suggests the occurrence of similar structural features for H12 positioning.

**DISCUSSION**

One generally accepted mechanism of antagonism is that steric hindrance inhibits the natural agonist conformation and favors an alternative position for H12 that then occupies the binding site of coactivators in the H3/H4 cleft. The present study allows us to dissociate the steric effect from the others. Indeed the crystal structure of the Cys→Ser triple mutant LBD bound to estradiol adopts a typical antagonist conformation, whereas estradiol binding and transactivation are not impaired. Each single Cys→Ser mutation lowers the transactivation activity by ~20%, showing that the structurally unrelated regions that are perturbed are equally important for EREs to fully respond to estradiol stimulation. The combination of these mutations results in an additive effect in the reduction of transactivation, leading to a triple mutant that is only half as potent as the wild type receptor. This cumulative effect of the Cys→Ser mutations further confirms the lack of cooper-
activity of the observed conformational changes. The higher amount of estradiol needed to stimulate transcriptional activity, compared with the lower concentration of raloxifen required to repress it, suggests that Cys → Ser mutations favor the switching of helix H12 toward a nonproductive conformation. These results favor a dynamic model where H12 occupies two more or less favorable states, with the mutations or the ligand affecting the equilibrium. This H12 flexibility is also illustrated by the ERβ bound to genistein complex (15). Genistein distinguishes itself from SERMs by its smaller size, allowing its total burying in the ligand binding cavity. In the crystal structure, helix H12 adopts a position shifted by 25° from the antagonist conformation observed in the raloxifen complex. Taken together with the highly disordered helix H12 in the ERβ-raloxifen crystal structure and similar observations in other systems (RXR-RAR), it is clear that antagonist positions are not unique. The RXRα F318A LBD-oleic acid complex (34), with a typical antagonist conformation for H12, bears even more resemblance to our present situation in that the ligand also does not prevent the agonist position. In this case too the antagonist conformation is in apparent contradiction with the transcriptional activity of the receptor and its ability to recruit coactivators (34). Furthermore an agonist position with a weak
transcriptional ability. When estradiol binds to ER, it interacts conformation, the equilibrium is shifted toward an alternative weakened or disrupted by altering the protein hydration and/or essential for stabilizing the agonist conformer. If these are mutant structures reveals the molecular interactions that are anchored by His524 that interacts with the near the mutated residues. The ligand is (5).

**CONCLUDING REMARKS**

The destabilization of the H12-protein core interaction is at the heart of the mechanism of partial and pure antagonism. The dominant effect depends on the potency of the ligand to disrupt the active conformation or in other words to prevent the correct binding of coactivators. The present study sheds light on the molecular mechanism and the structural basis of partial agonism on the AF2 transactivation function. The position of H12 can be modulated by the cellular context of cofactors, their ability to displace the equilibrium and to stabilize one conformer.

For the glucocorticoid and estrogen receptor, cysteine modification plays a role in gene regulation by the intracellular redox potential modification (36–38). The importance of cysteine residues located in the activation domain has recently been stressed for the nuclear factor I/CCAAT transcription factor (NFI/CTF) (39). Intracellular thioredoxin or metal ion concentration, which have high affinity for sulfhydryl groups, would act as the regulator of the transcription. Their effect on transactivation could be explained by the modification of cysteines, linking hormonal and redox signaling pathways.

ER, like other steroid nuclear receptors, is unstable in the absence of ligand or protein cofactors like HSP90 (33). The fold stabilization of these proteins is part of the control of gene expression and is ligand-dependent (induced fit mechanism) and controlled by the cellular context (redox potential, nature of a ligand, presence of interacting molecules like coactivators or corepressors).

**REFERENCES**

1. Mangelsdorf, D. J., and Evans, R. M. (1995) *Cell* 83, 841–850
2. Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (1996) *Chem. Biol. 3*, 529–536
3. McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999) *Endocr. Rev. 20*, 321–344
4. Ruff, M., Gangloff, M., Wurtz, J. M., and Moras, D. (2000) *Breast Cancer Res. 2*, 383–389
5. Moras, D., and Gronemeyer, H. (1998) *Curr. Opin. Cell Biol. 10*, 384–391
6. Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature 375*, 377–382

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**Fig. 5.** a, effect of C417S mutation on H3. Superposition of wild type (yellow) and Cys → Ser triple mutant (gray) emphasizing the shortening of H3 by one turn and the significant conformational change of the loop 1–3 are shown. b, effect of C530S mutation on H11. Superposition of wild type (yellow) and Cys → Ser triple mutant (gray), showing the shortening of H11 on the mutant protein is shown. c, superposition of wild type (yellow) and triple mutant (gray) ER LBD structures near the mutated residues. The ligand is (5).
