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Raloxifene and ICI182,780 Increase Estrogen Receptor-α Association with a Nuclear Compartment via Overlapping Sets of Hydrophobic Amino Acids in Activation Function 2 Helix 12

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The basis for the differential repressive effects of antiestrogens on transactivation by estrogen receptor-α (ERα) remains incompletely understood. Here, we show that the full antiestrogen ICI182,780 and, to a lesser extent, the selective ER modulator raloxifene (Ral), induce accumulation of exogenous ERα in a poorly soluble fraction in transiently transfected HepG2 or stably transfected MDA-MB231 cells and of endogenous receptor in MCF7 cells. ERα remained nuclear in HepG2 cells treated with either compound. Replacement of selected hydrophobic residues of ERα ligand-binding domain helix 12 (H12) enhanced receptor solubility in the presence of ICI182,780 or Ral. These mutations also increased transcriptional activity with Ral or ICI182,780 on reporter genes or on the endogenous estrogen target gene TFF1 in a manner requiring the integrity of the N-terminal AF-1 domain. The antiestrogen-specific effects of single mutations suggest that they affect receptor function by mechanisms other than a simple decrease in hydrophobicity of H12, possibly due to relief from local steric hindrance between these residues and the antiestrogen side chains. Fluorescence anisotropy experiments indicated an enhanced regional stabilization of mutant ligand-binding domains in the presence of antiestrogens. H12 mutations also prevent the increase in bioluminescence resonance energy transfer between ERα monomers induced by Ral or ICI182,780 and increase intranuclear receptor mobility in correlation with transcriptional activity in the presence of these antiestrogens. Our data indicate that ICI182,780 and Ral locally alter the ERα ligand binding structure via specific hydrophobic residues of H12 and decrease its transcriptional activity through tighter association with an insoluble nuclear structure. (*Molecular Endocrinology* 21: 797–816, 2007)

ESTROGENS, SUCH AS 17β-estradiol (E2), have pleiotropic actions on a number of target tissues in the skeletal, reproductive, cardiovascular, and central nervous systems (1–4). These actions are mediated by two estrogen receptors, ERα and ERβ (4, 5), members of the nuclear receptor superfamily of ligand-inducible transcription factors (6–9). Like other unliganded steroid hormone receptors, ERs are thought to interact in the absence of hormone with molecular chaperone complexes including the heat shock protein hsp90, the cochaperone p23, and immunophilins (10, 11). Hormone binding induces conformational changes resulting in binding to DNA (12–15) and in the ordered recruitment of a series of coactivator complexes responsible for histone acetylation, chromatin remodeling, and enhanced recruitment of the basal transcription machinery (16–21). Binding to DNA is achieved through specific interactions between the central DNA-binding domain, corresponding to homology region C (22, 23), and palindromic estrogen response elements [EREs (24–27)]. Two transcriptional activation functions are localized on either side of the DNA-binding domain. The activa-

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Abbreviations: AF-1, Activation function 1; AF-2, activation function 2; BRET, bioluminescence resonance energy transfer; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; FBS, fetal bovine serum; FRAP: fluorescence recovery after photobleaching; H12, helix 12; HEK, human embryonic kidney; HSB, high salt buffer; LBD, ligand-binding domain; LSM, laser scanning microscopy; MTMR, tetramethylrhodamine-5-maleimide; OHT, hydroxytamoxifen; PDB, protein database; Ral, Raloxifene; ROI, region of interest; RFU, relative fluorescence units; SERM, selective estrogen receptor modulator; TIF2, transcriptional intermediary factor 2; wt, wild type; YFP, yellow fluorescent protein.

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tion function AF-2 is located in the C-terminal ligand-binding domain (LBD, region E), and recruits coactivators in a ligand-dependent manner. The activation function AF-1, in the N-terminal A/B region, can function in a ligand-independent manner and is very variable both in length and sequence in the nuclear receptor superfamily (5, 6, 28, 29).

The observation that estrogen induces proliferation of mammary epithelial cells and of ERα-positive breast tumor cells has led to the development of antiestrogens for the treatment and prevention of breast cancer (30–32). Antiestrogens are competitive antagonists of estrogen and block the transcriptional activation properties of ERs. However, some antiestrogens display partial estrogenic activity in a tissue- and gene-dependent manner, hence their description as selective estrogen receptor modulators (SERMs). In animal models, both 4-hydroxytamoxifen (OHT) and raloxifene (Ral) have a favorable, estrogen-like action in bone (33). However, OHT has marked estrogenic activity on the rodent uterus, whereas Ral has only low activity in this model (33, 34). On the other hand, full antiestrogens such as ICI164,384, ICI182,780, and RU58,668 (35–37) completely block transcriptional activity of ERs in breast and uterine tissues.

Transcriptional activity of ERs in the presence of OHT has been observed in different cellular models and correlates with activity of the AF-1 region (38, 39). Recruitment of corepressors nuclear receptor corepressor (N-CoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT) in the presence of antiestrogens has been demonstrated (40–42), and it has been proposed that a higher degree of interaction with corepressors in the presence of full antiestrogens explains their more complete antagonist activity compared with OHT (43). However, effects on ERα protein turnover also provide another explanation for the different pharmacological properties of antiestrogens. OHT stabilizes the ERα protein (44, 45), whereas full antiestrogens induce a rapid loss of nuclear ERα, resulting in depletion of the receptor from estrogen-responsive promoters (15). Clearance of nuclear ERα correlates with proteasome-dependent degradation in ERα-positive cells (46–49). In addition, ERα was reported to accumulate in insoluble complexes in MCF7 cells in the presence of full antiestrogens and proteasome inhibitors (49). Formation of cytosolic aggregates was reported in transfected cells (44, 45, 50), whereas fluorescence recovery after photobleaching (FRAP) experiments performed in transfected HeLa cells have indicated slower intranuclear dynamics of ERα in the presence of ICI182,780 (51). These observations indicate a variety of potential mechanisms of receptor inactivation by full antiestrogens. Moreover, Ral has often more limited agonist activity than OHT in ERα-expressing cells or in transiently transfected cell lines (43, 52–56), but the mechanisms of its stronger repressive effects remain poorly characterized to date.

Antiestrogens have been shown by crystallography studies to bind to ERs in a manner similar to that of estrogens, but to prevent folding of the LBD into its agonist conformation due to steric hindrance of the antiestrogen side chain (57–59). In particular, helix 12 (H12), which is crucial for AF-2 activity, is displaced by the antiestrogen side chain from its position in the agonist conformation on top of the ligand binding cavity. The crystal structures of ERα complexed to antiestrogens OHT or Ral are similar, with H12 associating with the coactivator binding groove formed by helices H3–H5, thus preventing coactivator recruitment by AF-2 (57, 58). On the other hand, in the crystal structure of rat ERβ complexed to ICI164,384, the longer side chain characteristic of full antiestrogens (35–37) interacts directly with the coactivator binding groove (59). The position of H12 is undefined, suggesting conformational flexibility.

Specific mutations in H12 have been shown to convert the full antiestrogen ICI164,384 into an agonist in some experimental cell systems (44, 60–62), indicating the importance of H12 in the antagonist activity of antiestrogens. However, it remains unclear which residues of H12 contribute to the antagonist activity of full antiestrogens and which properties of the receptor are altered by these residues. In addition, it is currently unknown why Ral, which induces a structure of the ERα LBD similar to that observed with OHT, displays a degree of antagonist activity comparable to that of full antiestrogens in transfected cells. In this study, we have sought to analyze the molecular basis and mechanisms of the more pronounced antiestrogenic action of Ral and ICI182,780 *vs.* OHT using different cell models expressing the wt receptor or a series of point mutants affected in H12 residues.

RESULTS

Ral and Full Antiestrogens Decrease Extractability of ERα from a Nuclear Compartment in HepG2 Cells

HepG2 cells are a well-established model system for studying the differential activity of antiestrogens (14, 62–65). In these cells, cotransfected with an expression vector for human ERα and the ERE3-TATA-Luc reporter vector, transcriptional activity was observed in the absence of hormone, due to basal activity of the receptor, and was induced approximately 5-fold in the presence of estradiol. Saturating concentrations of OHT [sufficient to fully displace estradiol in competition experiments (data not shown)] were partially permissive for transcriptional activity of the reporter vector, whereas either the full antiestrogen ICI182,780 or the SERM Ral fully repressed the receptor transcriptional activation properties (Fig. 1A). Thus, in this model system, Ral behaves more like a full than a partial antiestrogen. In addition, similar results were obtained in transiently transfected HeLa cells, except that the degree of transcriptional activity obtained with OHT was smaller (data not shown).

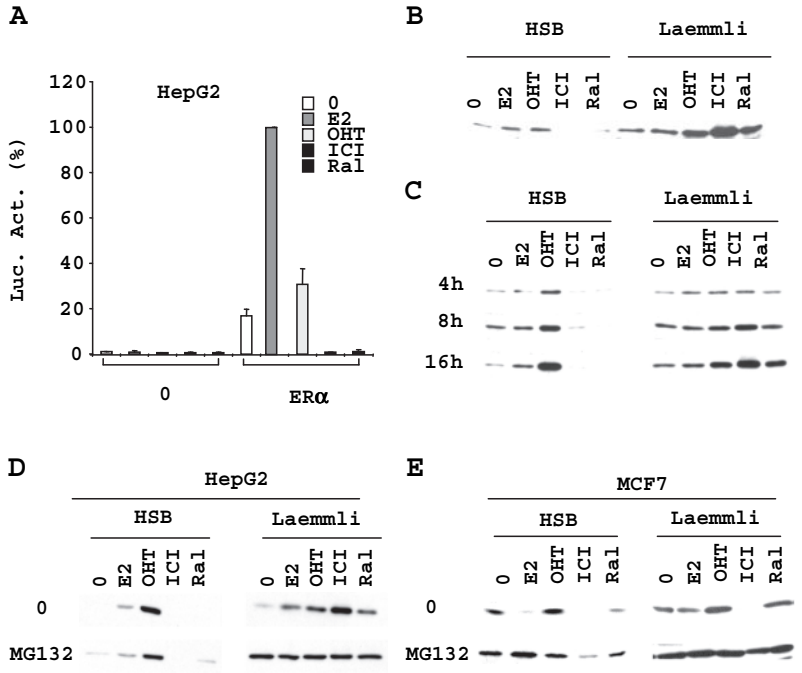

Fig. 1. The SERM Ral and the Full Antiestrogen ICI182,780 Both Efficiently Repress ERα Transcription and Decrease Soluble Receptor Levels in HepG2 Cells

A, Transient transfection experiments were performed in HepG2 cells by electroporation with an ERE3-TATA-Luc reporter, the internal control plasmid CMV-β-gal, and expression vectors for wt ERα or mutant ΔH12, as indicated. Cells were treated with vehicle (0), E2 (25 nM), OHT (100 nM), ICI 182,780 (ICI, 100 nM) or Ral (100 nM), for 48 h. Luciferase activity and β-galactosidase activity were quantified, and relative luciferase activity was calculated. Results from at least three experiments performed in triplicate are shown; *error bars* reflect the SD between independent experiments. B, After transient transfection with an expression vector for wt ERα, HepG2 cells were cultured in steroid-free medium for 36 h and subsequently treated with vehicle (0), E2, OHT, ICI182,780 (ICI), or Ral for 16 h. Extracts in HSB or Laemmli sample buffer (Laemmli) corresponding to one tenth of a plate in both cases were analyzed by SDS-PAGE and Western analysis using the anti-ERα antibody B10. C, A time course study of ERα protein levels after ligand treatment was performed by Western analysis as described in panel B. D, Receptor levels were evaluated in HepG2 cells transiently transfected with an expression vector for wt ERα and treated with hormones as in panel B. Treatment with the proteasome inhibitor MG132 (10 μM) was initiated 1 h before hormonal treatment. Extracts in HSB (50 μg) or Laemmli sample buffer were analyzed by SDS-PAGE and Western analysis using anti-ERα antibody B10. E, MCF7 cells were cultured in steroid-free medium for 36 h and subsequently treated with hormones and proteasome inhibitor as in panel D. Extracts in HSB (50 μg) or Laemmli sample buffer were analyzed by SDS-PAGE and Western analysis also as in panel D. Luc. Act., Luciferase activity.

To determine whether Ral and ICI182,780 induce receptor degradation in HepG2 cells, we performed a Western analysis of receptor levels in whole-cell extracts of HepG2 cells prepared using high-salt buffer (HSB). As reported before in a variety of cell systems (47–49, 66–69), OHT increased receptor levels compared with vehicle controls (Fig. 1B, *left panel*); estradiol also led to increased receptor content in the HSB fraction in HepG2 cells, but to lower levels than OHT (Fig. 1B, *left panel*). On the other hand, ICI182,780 induced disappearance of the receptor from the HSB-soluble fraction (Fig. 1B, *left panel*). In contrast to OHT, Ral did not lead to stabilization of the receptor but like ICI182,780 decreased receptor content in HSB extracts (Fig. 1B, *left panel*).

Because formation of cytoplasmic aggregates of ERα was observed in the presence of full antiestrogens in some cell systems (45, 50), we also examined receptor levels in Laemmli buffer, which extracts not only high-salt soluble receptor but also insoluble receptor aggregates. Western analysis performed with the same number of cells extracted either with HSB or Laemmli extract reveals that higher levels of receptor were extracted from cells with Laemmli buffer under all conditions, but that there was a marked accumulation of the receptor in the presence of ICI182,780 and, to a lesser extent, OHT or Ral (Fig. 1B, *right panel*). Comparison of profiles obtained with HSB and Laemmli extracts suggest therefore that disappearance of ERα from HSB extracts with ICI182,780 or Ral results from accumulation of the receptor in an insoluble form rather than from increased degradation. To determine whether insolubility of the receptor was due to formation of cytosolic aggregates in HepG2 cells, we performed an immunocytochemical analysis of ERα distribution on cells treated with vehicle, ICI182,780, or Ral for 16 h. Although cytosolic aggregates could be detected in some of the transfected cells treated with

ICI182,780 or Ral, this represented only a minority of the transfected cells (8% for ICI182,780 and 2.5% for Ral). Nuclear staining was observed in the majority of the transfected cells treated with antiestrogens as in cells exposed to vehicle only (Fig. 2).

To examine the kinetics of receptor insolubility in HepG2 cells, its distribution in the HSB-soluble and total (Laemmli buffer-soluble) fractions was determined at different times after exposure to antiestrogens. Poor extraction of the receptor in HSB was already observed at 4 h in the presence of ICI182,780 or Ral (Fig. 1C). Receptor levels gradually increased as a function of time in the presence of all antiestrogens in Laemmli extracts (Fig. 1C, *right panel*), but only in the presence of OHT in the HSB-soluble fraction (Fig. 1C, *left panel*). Together, these results suggest that all three antiestrogens stabilize the receptor against degradation in HepG2 cells, leading to a time-dependent accumulation reflecting *de novo* synthesis. However, these antiestrogens have markedly different effects on receptor solubility.

To verify that the variable levels of receptor observed both in HSB and Laemmli extracts from cells treated with the various ligands reflect different rates of degradation, we examined whether treatment of cells with the proteasome inhibitor MG132 would equalize levels of receptors under all treatment conditions. This was indeed the case in Laemmli extracts (Fig. 1D, *right panel*), confirming that all antiestrogens protect the receptor from degradation compared with no treatment or treatment with E2, but that ICI182,780 and Ral prevent extraction of the receptor in HSB.

Although these findings suggest different biophysical properties of the receptor in the presence of different antiestrogens, they contrast with reports that full antiestrogens induce receptor degradation in ER+ cells (47–49, 66–69). Indeed, under similar experimental conditions, treatment of MCF7 cells with ICI182,780 induced a depletion of receptor levels both in HSB and Laemmli extracts, although slightly higher levels of receptor were detected in Laemmli extracts (Fig. 1E, *upper panels*). Treatment of cells with MG132 fully restored receptor levels to amounts observed in the absence of treatment in Laemmli extracts (Fig. 1E, *lower right panel*), indicating that the receptor is indeed degraded in the presence of ICI182,780. However, receptor levels were still reduced by treatment with ICI182,780 in HSB even in the presence of MG132, indicating that the endogenous receptor in MCF7 cells is insoluble in the presence of ICI182,780 as well as in HepG2 cells. This property may explain the observation that treatment with proteasome inhibitors does not increase transcriptional activity of the receptor in the presence of ICI182,780 (data not shown). In MCF7 cells, receptor levels in the presence of Ral in the HSB fraction were intermediate between those with OHT and ICI182,780 and were increased either by extraction in Laemmli or by treatment with MG132, suggesting that both degradation and insolubility of the receptor contribute to reduced levels in the presence of Ral. In addition, ERα accumulated in an insoluble fraction in MCF7 treated with ICI182,780 or Ral at early time points (20 min) before degradation is initiated (data not shown). In conclusion, the overall patterns of receptor levels in the presence of antiestrogens are similar in HepG2 and MCF7 cells, and insolubility of the receptor in the presence of full antiestrogens is not restricted to transiently transfected cells.

Specific Long Hydrophobic Amino Acids of H12 Play a Role in Transcriptional Repression and Insolubility of the Receptor in the Presence of Ral or ICI182,780

A major difference in the structures of the receptor complexed to SERMs and full antiestrogens is the position of H12, which is present at the C terminus of the LBDs of all nuclear receptors (Fig. 3, A and B). H12 is amphipathic (Fig. 3C) and its hydrophobic face is buried against the rest of the LBD in the presence of agonists or of SERMs. Indeed, H12 acts as a lid to the ligand binding cavity in the presence of agonists. On the other hand, it binds to the coactivator binding groove in the presence of OHT or Ral, residues 540, 543, and 544 mimicking the critical leucine residues in the coactivator LXXLL motifs (57, 58). In contrast, the coactivator binding groove position is occupied by the long side chain of the full antiestrogen ICI164,384, and the position of H12 is not defined in the crystal structure with ERβ, suggesting conformational flexibility (59).

Although the positioning of helix 12 is similar in the presence of OHT and Ral (57, 58), we decided to investigate the role of this helix in antiestrogen-induced insolubility of the receptor based on previous studies reporting that combined mutation to alanines of two hydrophobic amino acids (L539-L540 and M543-L544), or the single L540Q mutation in H12 can lead to receptors with an inversion in their patterns of transcriptional activity with agonists and antagonists in transfected cells (44, 60–62). To investigate whether amino acids of H12 play a role in accumulation of the receptor in an insoluble fraction in the presence of Ral and/or ICI182,780 in HepG2 cells, and clarify the relationship between this property and transcriptional repression, we performed an alanine scanning mutagenesis of all hydrophobic amino acids in H12. Note that although amino acids 536–537 are not part of H12 in the agonist conformation, they are incorporated into H12 in the presence of SERMs (57, 58) and therefore were included in this analysis.

The activity of the different mutants was tested in HepG2 cells in the presence of agonists and antagonists (Fig. 4A). Contrary to the wt receptor, several mutants (at positions 536, 539, 540, 543, and 544) were transcriptionally active in the presence of ICI182,780. Note that titration curves performed with mutant L536A, which had a higher degree of activity in

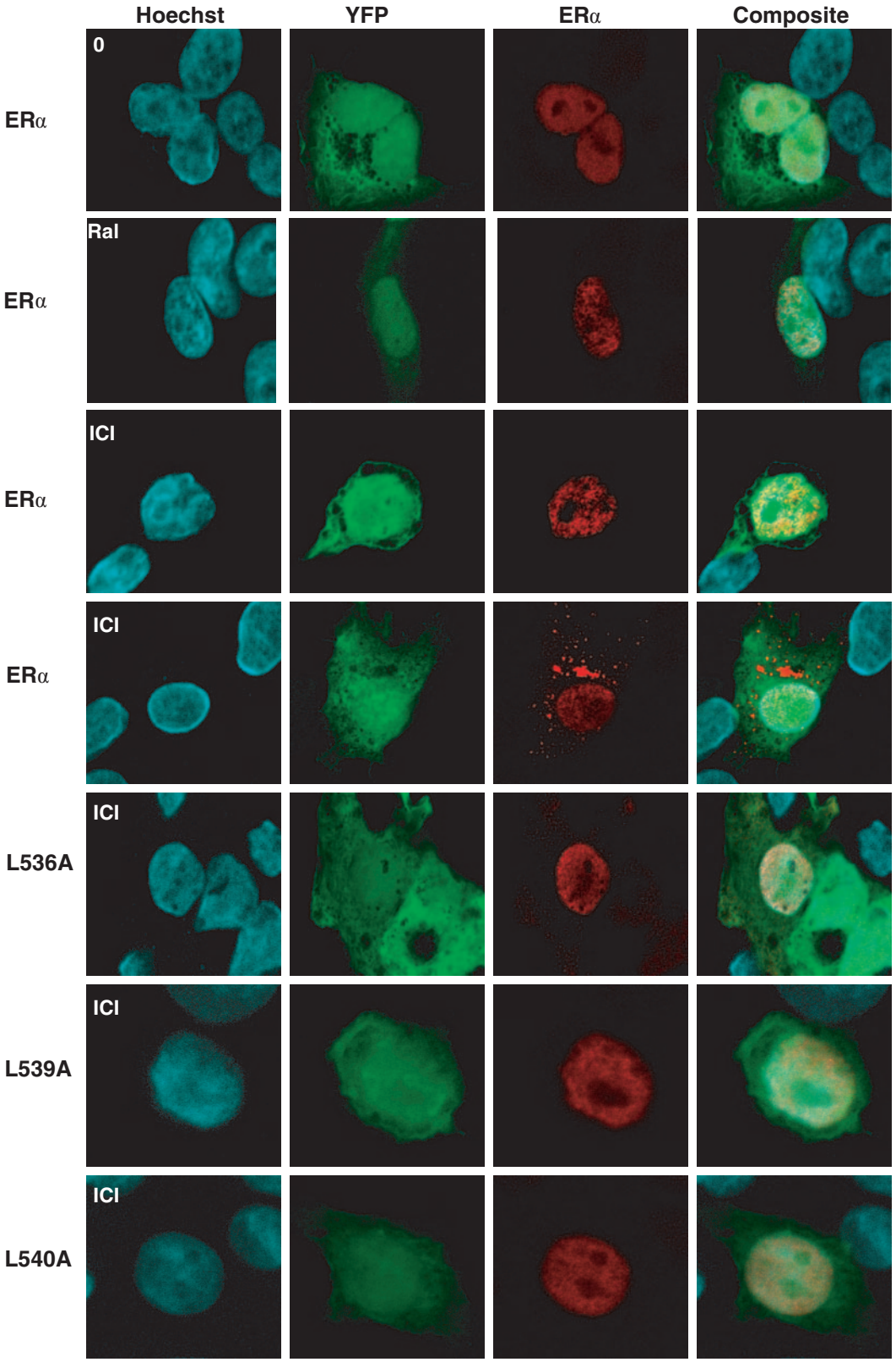

Fig. 2. Nuclear Localization of wt ERα and of H12 Mutant Receptors in HepG2 Cells

HepG2 cells were cotransfected with expression vectors for wt ERα or mutants affected in H12 (as indicated) and for YFP, and were treated with vehicle or ligands for 16 h before fixation, permeabilization, and antibody staining with a mouse anti-ERα (B10) and an Alexa Fluor 595 dye-labeled goat antimouse secondary antibody. Images are from *left* to *right*: nuclear staining (Hoechst 33342), YFP, ERα, and composite images. ICI, ICI182,780.

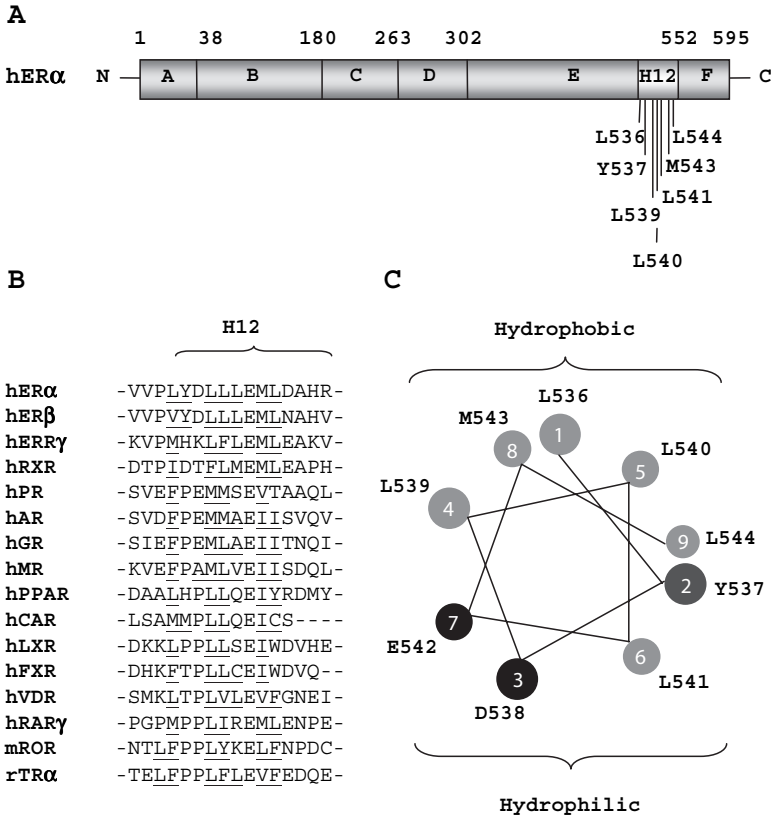

Fig. 3. Conservation of Hydrophobic Amino Acids in H12 in the Nuclear Receptor Superfamily

A, The domain organization characteristic of nuclear receptors is shown, and amino acids at the interdomain boundaries in ERα are indicated. The hydrophobic amino acids in helix H12 of the LBD are also highlighted. B, Alignment of H12 and flanking sequences (residues 535–545 in ERα) in the nuclear receptor superfamily. Conserved hydrophobic amino acids are *underlined* (h, human; r, rat; m, murine). C, Positioning of the hydrophobic residues in H12. Nonpolar (*light gray*), polar and uncharged (*medium gray*), or acidic (*black*) residues are placed on an α-helical wheel (generated using software at http://cti.itc.virginia.edu/~cmg/Demo/contents.html). AR, Androgen receptor; CAR, constitutive androstane receptor; ERR, estrogen-related receptor; GR, glucocorticoid receptor; LXR, liver X receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; RAR, retinoic acid receptor; RXR, retinoic X receptor; VDR, vitamin D receptor.

the absence of ligand than in the presence of ICI182,780, confirmed that the increased activity in the presence of ICI182,780 was not due to lack of saturation of the receptor (data not shown). Activity in the presence of ICI182,780 was significantly increased with mutants L536A, L539A, L540A, M543A, and L544A compared with the response of the wt ERα ($P <$ 0.01 in Student's *t* test). A subset of these mutations also increased activity in the presence of Ral (536, 539, and to a lesser extent 544; $P < 0.01$). Interestingly, whereas mutants at positions 536 and 539 were more active with Ral than ICI182,780, mutant L540A had the opposite activity profile. Increased activity in the presence of antiestrogens was observed with mutants that had normal as well as reduced levels of estrogen-induced transcription. Similarly, there was no correlation between levels of basal activity, which were either reduced or increased by these mutations, and levels of activity in the presence of antiestrogens. This suggests that molecular determinants of activity in the presence of antiestrogens differ from those controlling transcriptional activity in the absence of ligand or in the presence of agonists. Finally, note that not all mutations in long hydrophobic residues generated increased activity of the receptor in the presence of Ral or ICI182,780, because mutants Y537A and L541A had an activity profile similar to that of wt receptor.

To assess whether mutations that increased activity in the presence of antiestrogens have altered solubility profiles, we performed Western analyses on all above described ERα mutants. Receptor levels in the soluble fraction in the presence of ICI182,780 or Ral correlated well with transcriptional activity of the receptors (Fig. 4B). All mutants with increased activity in the presence of Ral (L536A, L539A, and, to a lesser extent, L544A) were present at markedly increased levels in high-salt extracts. In particular, protein levels and transcriptional activity were both as high in the presence of Ral as of OHT for the L536A and L539A mutants (Fig. 4, A and B). In addition, mutants with increased activity in the presence of ICI182,780 (L536A, L539A, L540A, M543A, and L544A) were all detected at higher levels

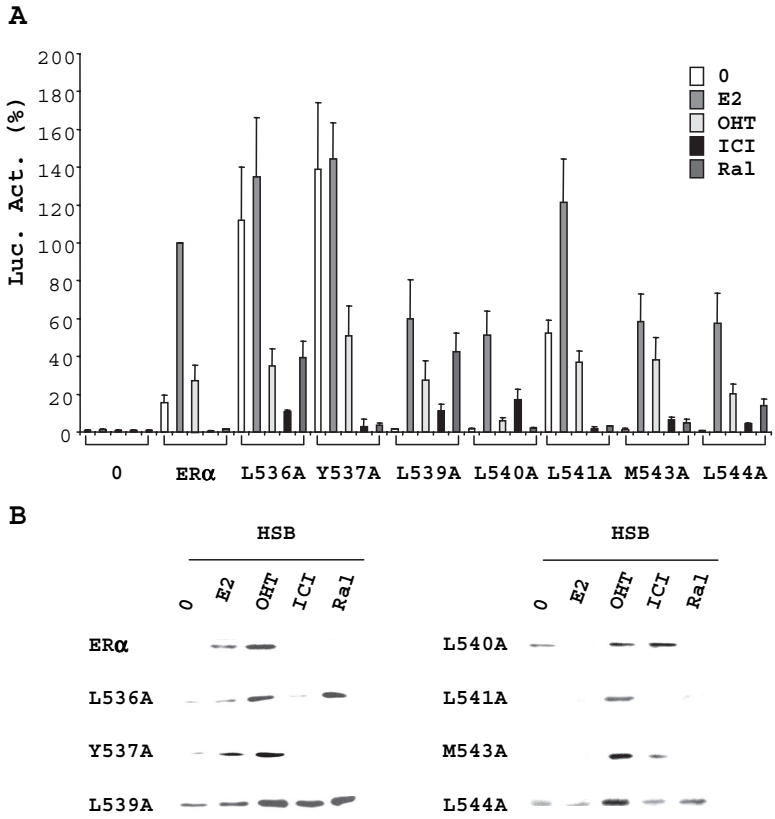

Fig. 4. The Long Hydrophobic Amino Acids of H12 Modulate Receptor Solubility and Activity in an Antiestrogen-Specific Manner

A, HepG2 cells were electroporated with the ERE3-TATA-Luc reporter, the internal control plasmid CMV-β-gal, and expression vectors for wt ERα or mutants affected in H12 hydrophobic residues. Cells were treated with hormones and harvested as described in Fig. 1A. Relative luciferase activity is shown. B, After transient transfection with expression vectors for wt or mutant ERα, HepG2 cells were cultured in steroid-free medium for 36 h and subsequently treated with hormones as in Fig. 1B. HSB extracts (HSB) (50 μg) were analyzed by SDS-PAGE and Western analysis using anti-ERα antibody B10 as described in Fig. 1B. ICI, ICI182,780.

in HSB extracts than the wt receptor in the presence of this antiestrogen (Fig. 4B). Notably, mutant L540A had higher solubility in the presence of ICI182,780 than Ral (Fig. 4B), correlating with the transcriptional profiles in the presence of these antiestrogens (Fig. 4A). Finally, mutants Y537A and L541A had similar patterns of extraction in HSB as the wt receptor, as well as similar patterns of transcriptional activation, in the presence of OHT, Ral, and ICI182,780.

Together, these observations suggest that mutations in H12 affect simultaneously receptor extractability from the nuclear compartment and transcriptional activity in the presence of antiestrogens ICI182,780 or Ral.

Partial Agonist Activity of Antiestrogens on the TFF1 Target Gene in MDA-MB-231 Cells Stably Transfected with the L539A Mutant

Although profiles of receptor levels in the presence of antiestrogens were similar in MCF7 and transiently transfected HepG2 cells, it remains possible that high expression levels generated in transfected HepG2 cells may lead to artifactual insolubility and not reflect the functional properties of receptors expressed at lower levels. To exclude this possibility, we generated stable cell lines expressing the wt ERα or mutant L539A in ERα-negative MDA-MB-231 cells. Hygromycin-resistant clones were analyzed for receptor expression by RT-PCR, and two clones with comparable expression levels were selected as well as a negative control clone propagating the empty parental vector (data not shown). Western analysis indicated that the expression levels of the wt receptor in the absence of hormone were lower than that of the endogenous receptor in MCF7 cells and that the pattern of ER levels in the presence of various antiestrogens after extraction in HSB was similar to those observed in MCF7 cells and HepG2 cells (Fig. 5A). The patterns of mutant L539A expression in HSB extracts were also comparable to those observed in HepG2 cells, with a relative increase in receptor levels in the presence of ICI182,780 and Ral compared with the wt receptor (Fig. 5B). Transient transfection of the ERE3-TATA-Luc reporter vector in the stable clones also indicated that

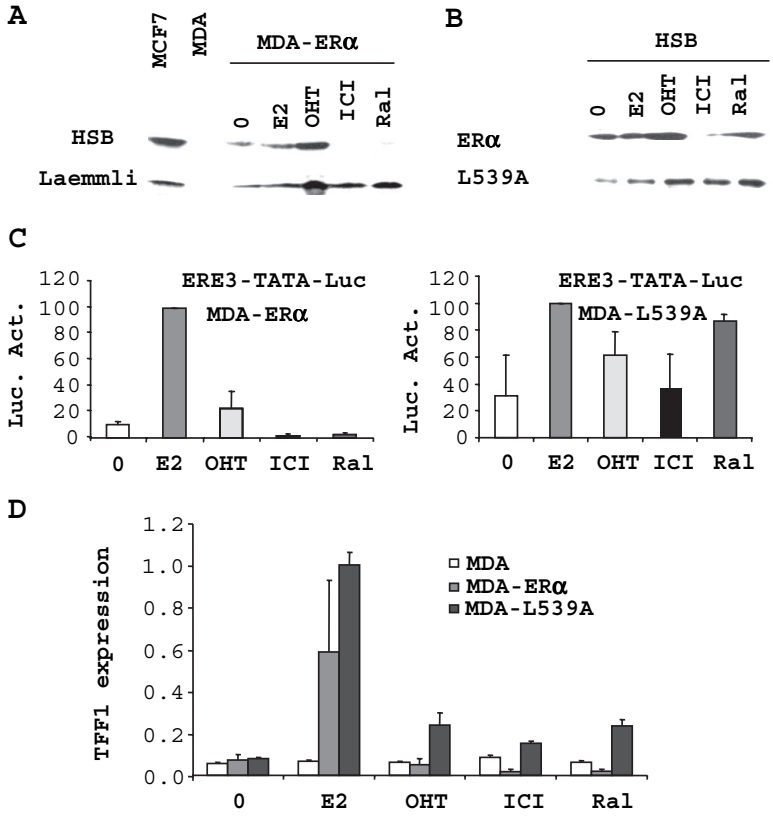

Fig. 5. The L539A Mutant Has Increased Transcriptional Activity in the Presence of Antiestrogens on the Endogenous pS2 Target Gene

A, Receptor levels in a stably transfected MDA-MB-231 clone expressing ERα (clone 22A) were determined both in HSB and Laemmli extracts using the B10 monoclonal antibody. Cells were treated with vehicle (0), E2, OHT, ICI182,780 (ICI), or Ral for 16 h and whole-protein extracts were prepared in either extraction buffer. Extracts from MCF7 or MDA-MB-231 cells were loaded for comparison. B, Western analysis of receptor levels in stably transfected MDA-MB-231 clones expressing ERα (clone 22A) or L539A (clone 4A) after hormonal treatment for 16 h and extraction in HSB. C, Transcriptional activity of the wt ERα or the L539A mutant was analyzed in stable clones (clone 22A and 4A, respectively) by transient transfection of the ERE3-TATA-Luc reporter construct. D, Transcription levels of the endogenous pS2/TFF1 gene in MDA-MB-231 stable clones were measured by quantitative-PCR amplification of reverse transcribed RNAs and standardized on expression of the housekeeping p36b4 gene, the mRNA levels of which are not affected by hormonal treatment. Luc. Act. Luciferase activity.

the patterns of transcriptional activity of the wt and mutant receptors were comparable to those observed in HepG2 cells. Indeed, ICI182,780 and Ral fully repressed activity of the reporter vector in the clone expressing the wt receptor, whereas they were partially permissive for transcription in the L539A clone, resulting in similar levels of activity with OHT and Ral, and slightly lower activity with ICI182,780 (Fig. 5C). Thus, results obtained in HepG2 cells are reproducible in cells that express receptors at near-endogenous levels.

Next, we investigated whether the L539A mutation could increase activity on endogenous receptor target genes. The pS2/TFF1 gene is a well-characterized estrogen target gene that contains a classical estrogen response element (70). Transcription of the TFF1 gene is returned to basal levels by OHT but further repressed by ICI182,780 and Ral (Fig. 5D; compare levels of activity obtained in the control and wt ERα-expressing clones). Basal levels of expression were similar in the clone expressing the L539A mutant and in the two other clones. On the other hand, levels of transcription of the TFF1 gene in the presence of ICI182,780 and Ral were significantly higher in the L539A clone than in the wt ERα-expressing clone ($P < 0.01$ in a *t* test). These results indicate that mutations in amino acids of H12 can lead to a gain in transcriptional activity in the presence of antiestrogens on endogenous estrogen target genes as well as with a reporter containing a minimal ERE3-TATA promoter.

Mutations in Long Hydrophobic Amino Acids of H12 Relieve Local Steric Hindrance with the Antiestrogen Side Chains

Because some mutations in hydrophobic residues (Y537A, L541A) had no effect on receptor solubility/activity and others had antiestrogen-selective effects, our results suggest a specific role of individual H12 residues on receptor conformation in the presence of

Ral or ICI182,780. To investigate this hypothesis, we superimposed complexes obtained in the presence of OHT (58), Ral (57), or ICI164,384, a compound closely related to ICI182,780 (59), to the ERα-E2 complex (71) and assessed the impact of antiestrogen binding on the agonist structure of the receptor. The side chains of antiestrogens created steric clashes with H12 in the agonist position at amino acids L536 (OHT; Fig. 6B), L540 (OHT, Ral, and, to a lesser extent, ICI164,384; Fig. 6, B–D) and/or M543 (ICI164,384; Fig. 6D). Depending on the extent of the clash, replacement of these residues by alanines may directly relieve steric hindrance. For instance, steric conflict between L540 and the side chain of ICI164,384, but not the more extensive overlap with the side chain of Ral, can be relieved by mutation to alanine (Fig. 6, C and D), correlating with a gain in transcriptional activity in the presence of ICI182,780, but not Ral, for this mutant. Replacement of L536 by alanine was also insufficient to relieve the steric clash with the OHT side chain and did not generate increased levels of transcriptional activity in the presence of this antiestrogen. Note that mutations removing the long hydrophobic side chains of amino acids close to those in direct steric conflict may result in rearrangement of the side chain of Ral or ICI182,780 in a manner that allows positioning of H12 in an agonist-like conformation.

We also examined the effect of the Ral or ICI164,384 side chains on positioning of H12 in the coactivator binding groove by superimposing the corresponding structures (57, 59) with that of OHT-bound ERα (Fig. 7, A and B) (57). The side chain of Ral was not found to generate important steric conflicts with H12, because the closest amino acids, L536 and L539, could form van der Waals contacts (Fig. 7C) as observed in the crystal structure of ERα with Ral (57). The ICI164,384 side chain, on the other hand, led to steric clash with L536, and less critical hindrance with L540 (Fig. 7D), amino acids the replacement of which by alanine residues increases agonist activity of ICI182,780.

In conclusion, many of the alanine mutations studied here reduce, either directly or indirectly, steric hindrance between antiestrogen side chains and H12 positioned either on top of the ligand binding cavity or in the coactivator binding groove and may thus increase LBD stability.

Increased Transcriptional Activity in the Presence of Ral or ICI182,780 Does Not Correlate with Increased Recruitment of an LXXLL Motif and Necessitates the Presence of the AF-1 Region

To examine whether mutations in several long hydrophobic amino acids of H12 result in gain in AF-2 activity in the presence of Ral or ICI182,780, we assessed recruitment of an LXXLL peptide in a mammalian two-hybrid assay (72). As expected, the α/βI peptide was recruited to the wt receptor only in

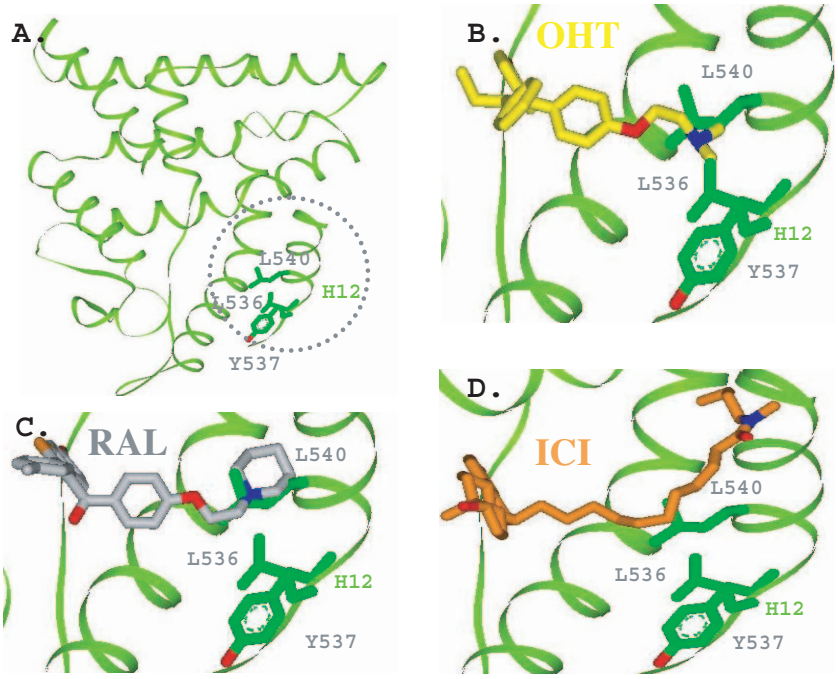

Fig. 6. Structural Constraints Exerted by the Side Chains of Antiestrogens on Positioning of H12 on Top of the Ligand Binding Pocket

The ERα-E2 complex (71) (A), was superimposed with that obtained in the presence of OHT (58) (B), Ral (57) (C) or ICI164,384 (59) (D) using the Lsq-man module of the O package. The side chain of Y537 does not result in steric hindrance with the antiestrogen side chains, whereas that of amino acid L536 or L540 result in steric clash with the side chain of OHT or Ral/ICI164,384 (ICI), respectively.

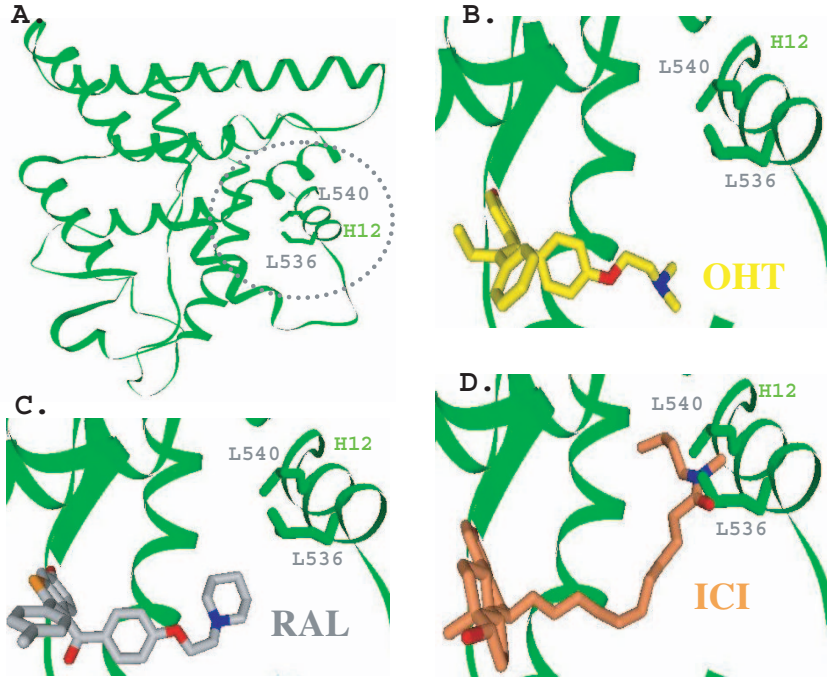

Fig. 7. Structural Constraints Exerted by the Side Chains of Antiestrogens on Positioning of H12 in the Coactivator Binding Groove

The structure of the ERα-OHT complex (58) (A and B) was superimposed with that obtained in the presence of Ral (57) (C) or ICI164,384 (59) (D) as in Fig. 6. The side chains of L536 and L540, which generate steric hindrance with the ICI164,384 (ICI) side chain, are shown.

the absence of ligand or the presence of E2, but not in the presence of any antiestrogen (Fig. 8). Recruitment in the presence of E2 was not drastically affected by the L536A, Y537A and L541A mutants, all of which transactivated an ERE3-TATA-Luc reporter vector at least as well as the wt receptor. Decreased E2-dependent recruitment was observed with the mutants affecting amino acids 539, 540, 543, and 544, which are known to be involved in stabilization of H12 in the agonist position and/or in interactions with the coactivator LXXLL motif (57, 58). This effect is consistent with the decrease in transactivation capacity observed with these mutants (Fig. 4). No recruitment of the LXXLL peptide was observed in the presence of either Ral or ICI182,780 with any of the mutants that had increased transcriptional activity with these antiestrogens, *i.e.* L536A, L539A, L540A, M543A, and L544A (Fig. 8). Similar results were obtained using a bioluminescence resonance energy transfer assay (data not shown). These observations indicate that the conformation of ERα mutants with increased agonist activity in the presence of Ral or ICI182,780 does not result in detectable AF-2 activity in this assay and suggest involvement of additional functional determinants in the observed gains in transcriptional activity.

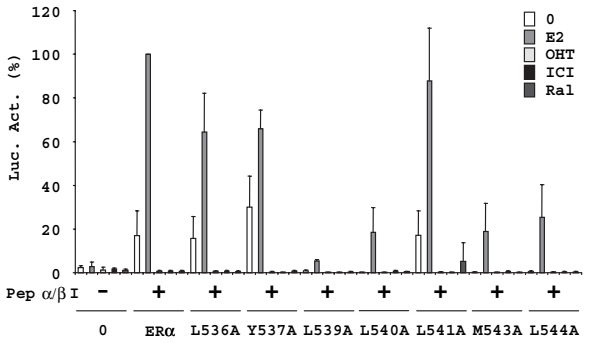

Fig. 8. Lack of Detectable Recruitment of an LXXLL Motif by Receptor Mutants with Increased Transcriptional Activity in the Presence of Ral or ICI182,780

HeLa cells were electroporated with the 5×GAL4-TATA-Luc reporter, the internal control plasmid CMV-β-gal, and expression vectors for the GAL4-pep α/β I fusion protein and for full-length wt ERα or mutants of H12 fused to VP16. Cells were treated with hormones and harvested as described in Fig. 1. Relative luciferase activity is shown. Luc.Act., Luciferase activity; Pep, peptide.

Because transcriptional activity in the presence of the partial antiestrogen OHT has been correlated with activity of the AF-1 region (38, 39), we have assessed whether this is also the case for activity of ERα mutants in the presence of full antiestrogens. In HepG2 cells, removal of the AF-1-containing AB region (ΔAB construct) practically inactivates the wt receptor, with residual activity detectable only in the presence of E2 on the minimal ERE3-TATA promoter. This is likely due to the loss of cooperativity between AF-1 and AF-2 for

coactivator recruitment (73). Transactivation in the presence of E2, but not in the presence of OHT, can be rescued by cotransfection of the core domain of coactivator TIF2 [transcriptional intermediary factor 2 (core domain TIF2.1)], member of the p160 family of coactivators (74, 75). Removal of the AF-1 activation function in mutant L536A, which displays increased levels of transcriptional activity in the presence of both ICI182,780 and Ral as well as in the absence of ligand, also led to loss of detectable activity in the presence of all ligands except for residual transcription in the presence of E2 (Fig. 9). Although TIF2.1 expression could restore activity in the presence of E2 and the absence of ligand, it did not enhance the activity of the truncated receptor ΔAB/L536A in the presence of OHT, ICI182,780, or Ral. Overexpression of the full-length steroid receptor coactivator 1 (SRC1), another member of the p160 family, also led to a partial rescue of activity in the presence of E2 or the absence of ligand, but failed to rescue activity in the presence of antiestrogens (data not shown). Similar results were obtained with ΔAB/L539A (data not shown). Together, these results strongly suggest that the AF-1 region is required for cofactor recruitment mediating the agonist activity of Ral and ICI182,780 with mutant receptors as well as for that of OHT with the wt receptor.

Mutations L536A and L539A Lead to a Selective Local Stabilization of the Receptor LBD in the Presence of Antiestrogens

To better investigate the molecular basis of the increase in ERα activity in the presence of antiestrogens resulting from mutations in long hydrophobic amino acids of H12, we examined regional LBD dynamics in two of these mutants, L536A and L539A, using a fluorescence polarization assay. By attaching an appropriate fluorophore, such as tetramethylrhodamine-5-maleimide, site-specifically at C530, we were able to develop a sensitive method for evaluating, in a rapid and quantitative manner, distinctive changes in the regional dynamics of the ERα LBD induced by the binding of ligands of a different pharmacological nature (76). Indeed, C530 is part of H11 in the LBD of ERα complexed to E2, but the alternative positioning of H12 induced by antiestrogens results in a different degree of helicity at this position, H11 terminating at position 530 in the presence of E_2, 529 for ICI182,780, 528 for Ral, and 526 for OHT. In the case of OHT or Ral, which have the shortest H11, this partial unwinding of H11 is necessary to allow repositioning of H12 in the coactivator binding groove (57, 58). The fluorescence signal shows higher anisotropy values when the local protein environment is rigid or α-helical (as in the agonist or ICI182,780 complexes) and lower anisotropy values when it is more dynamic or in a loop structure (as in the OHT complex).

As reported previously and also shown in Fig. 10, there are very significant differences ($P < 0.001$) between the anisotropy value of wt ERα complexed with E2 and the values with the Ral and OHT complexes, reflecting differences in the local conformation in the C530 region for these complexes (76). Interestingly, whereas the anisotropy values for unliganded and E2-bound L536A and L539A mutants were not markedly different from those of wt ERα, these mutations had a large effect on the anisotropy of the OHT, ICI182,780, and Ral complexes compared with those with wt ERα ($P < 0.001$). The anisotropy values for the Ral and ICI182,780 complexes with these mutants rise to the point that they are comparable to or greater than those of the corresponding E2-bound complexes, respectively; the anisotropy values for the two ERα mutants bound to OHT also increased significantly ($P < 0.01$). Thus, the mutational changes in L536A and L539A reduced conformational mobility of the C530 position

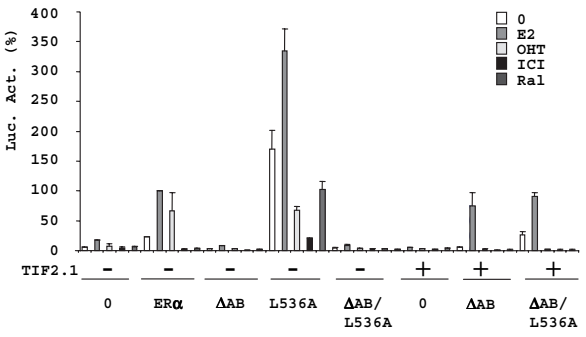

Fig. 9. Transcriptional Activity of ERα in the Presence of Ral or ICI182,780 Requires AF-1 Activity

Transient transfection analysis of the transcriptional activity of the wt ERα, or the mutant L536A and of derivatives thereof carrying deletions of the AB region in the presence or not of overexpressed TIF2.1 (4 μg) was performed as in Fig. 1. Relative luciferase activity is shown. Luc. Act., Luciferase activity.

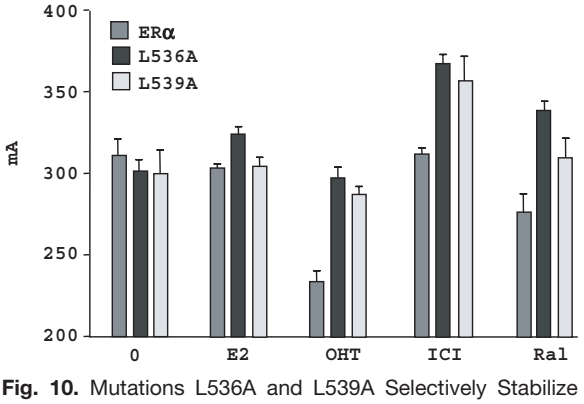

Fig. 10. Mutations L536A and L539A Selectively Stabilize Receptor Conformation in the Vicinity of C530 in the Presence of Antiestrogens

The ligand-induced regional dynamics of the LBDs of wt ERα, or of the L536A or L539A mutants were determined through fluorescence anisotropy assays (mA = anisotropy × 1000). Each fluorescence anisotropy value represents the mean ± SEM obtained from four independent experiments. ICI, ICI182,780.

considerably in the presence of all three antiestrogens, consistent with a more agonist-like conformation, although the conformational dynamics of each complex remained different.

ICI182,780 and Ral Induce Increases in Bioluminescence Resonance Energy Transfer between wt, but Not H12 Mutant Receptors in Live HepG2 Cells

To assess whether the different properties of the receptor in the presence of ICI182,780 and Ral *vs.* other ligands could be detected *in vivo* as well as through differential extraction or *in vitro* assays, we constructed fusion proteins between wt or mutant receptors and either *Renilla* luciferase or yellow fluorescent protein (YFP) and performed bioluminescence resonance energy transfer (BRET) assays between two wt or mutant ER monomers to determine whether antiestrogens affected receptor association and/or conformation. Titration curves performed with varying concentrations of the YFP fusion protein indicated a saturable autoassociation of the receptor in the presence of estradiol, ICI182,780, or Ral, with apparent affinities that were comparable for ICI182,780 and E2, and slightly lower for Ral (Fig. 11A; compare the slopes of the linear parts of the curves). These results indicate that the two antiestrogens do not have a destabilizing effect on the whole LBD structure. However, the maxima reached in the presence of the two antiestrogens were much higher than with E2 (Fig. 11A) or vehicle (data not shown; see also Fig. 11B), indicating a more efficient transfer of energy at saturation. Of interest, OHT did not induce an increase in energy transfer between wt receptors, and ICI182,780 or Ral did not increase energy transfer between mutant receptors that have increased solubility in the presence of these antiestrogens (Fig. 11B). These results suggest that the differential properties of antiestrogens in this BRET assay are linked to their different effects on receptor extractability. The increased energy transfer observed between wt but not mutant receptors could reflect a different conformation of the receptors bringing the YFP and luciferase moieties in closer proximity, and/or a higher local concentration of receptors, allowing exchange between one luciferase and several YFP molecules.

Mutations in H12 Increase Intranuclear Mobility of ERα in the Presence of ICI182,780 and Ral

It has previously been observed that the full antiestrogen ICI182,780 reduces the intranuclear mobility of ERα in transiently transfected HeLa cells (51). Because we also observe insolubility of ERα in HSB buffer in ICI182,780-treated HeLa cells (data not shown), we assessed whether insolubility of the receptor in HepG2 cells in the presence of ICI182,780 or Ral correlates with lower receptor mobility. FRAP experiments were performed with HepG2 cells transiently transfected

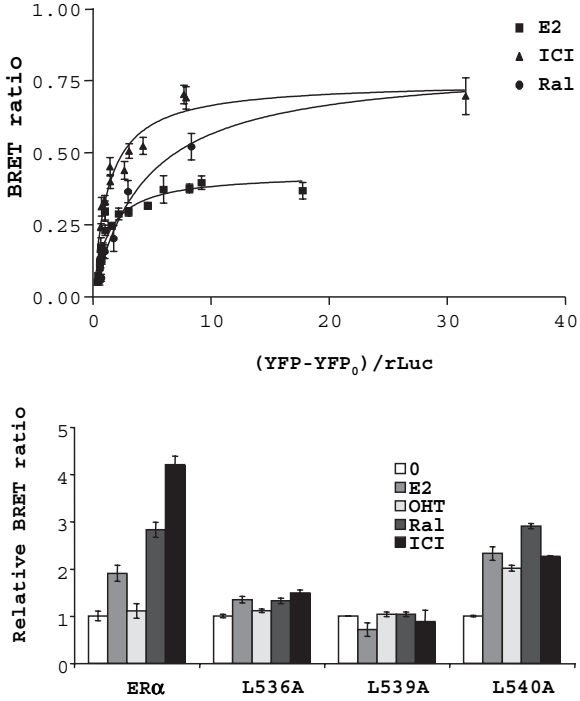

Fig. 11. Mutations Increasing ERα Solubility in the Presence of ICI182,780 or Ral Prevent Induction by These Antiestrogens of BRET between ER Monomers

HEK293 cells were transfected by expression vectors for fusion proteins between wt or mutant ERα and C-terminally fused rLuc or YFP proteins and treated for 40 min with different ligands. BRET ratios were calculated as described in *Materials and Methods*. A, Energy transfer between wt ERα monomers was measured using increasing concentrations of ERα-YFP fusion protein in the presence of E2, ICI182,780, or Ral. The x-axis value represents the ratio of ER-YFP fusion protein to ER-Luc fusion proteins, calculated as described in *Materials and Methods*. B, Effect of mutations in long hydrophobic amino acids of H12 on BRET ratios at saturating ratios of YFP *vs.* rLuc (*Renilla* luciferase) fusion proteins.

with an expression vector for the wt receptor fused to yellow fluorescent protein (YFP). The results indicate that treatment of cells with ICI182,780 or Ral prevents recovery of the fluorescence in the bleached zone over the course of the experiment (210 sec), with only a small fraction of the fluorescence being reconstituted (Fig. 12, *middle* and *bottom panels*). In contrast, fluorescence is nearly completely recovered with the unliganded receptor (Fig. 12, *top panel*). These observations are consistent with the immobilization of the receptor observed in HeLa cells in the presence of ICI182,780 and extend these observations to another cell context and to Ral.

To determine the impact of mutations in H12 on receptor mobility, we measured fluorescence recovery after bleaching in cells expressing fusion proteins with mutants L536A, L539A, and L540A (Fig. 12, *middle* and *bottom panels*). Note that mutations in long hydrophobic amino acids of H12 did not alter the nuclear distribution of the receptor in the presence of anties-

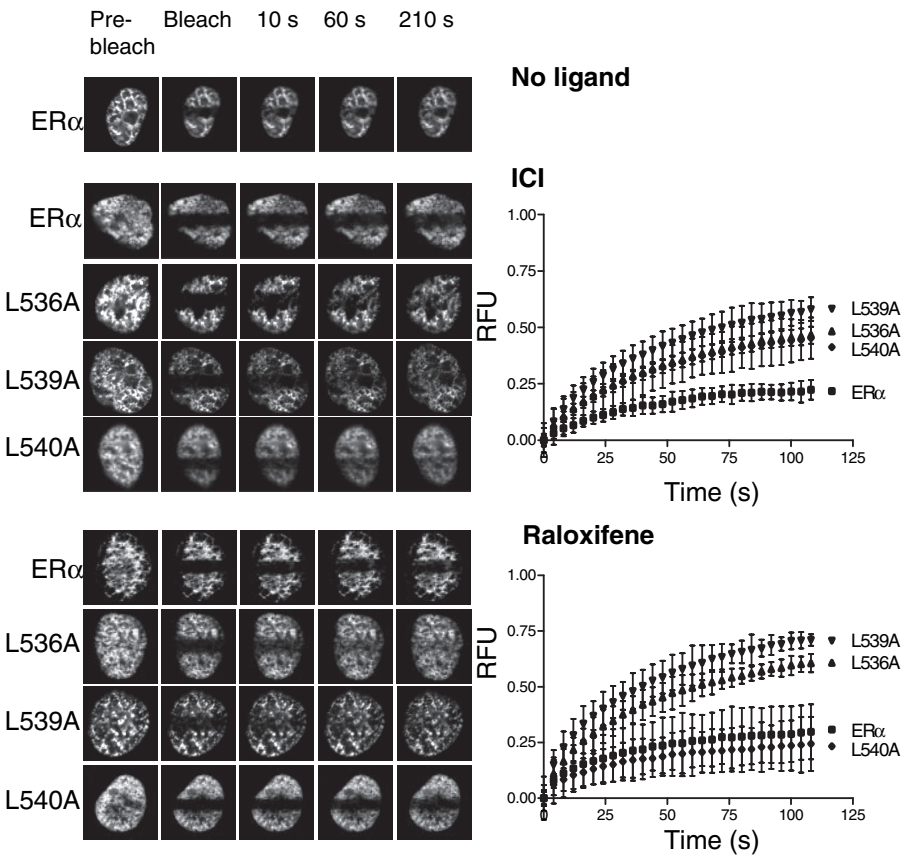

Fig. 12. Mutations Increasing ERα Solubility in the Presence of ICI182,780 or Ral Enhance Receptor Intranuclear Mobility

HepG2 cells transiently transfected with expression vectors for wt or mutant ERα fused to YFP were treated with vehicle or ligands for 16 h. A single region of interest was bleached with an Ar 488 laser. Images were captured at time intervals of 4 sec and analyzed for fluorescence intensities in the region of interest and in the whole cell by Meta Imaging Series 6.1 software. Fluorescence is represented in RFUs where 0 is the fluorescence after bleaching (time 0) and 1.00 is the expected fluorescence at homogeneity. *Error bars* represent the SD between three independent measurements. s, Seconds.

trogens ICI182,780 or Ral (data not shown). Strikingly, all mutations led to an increase in the fraction of the receptor that is mobile in the presence of ICI182,780. In addition, mutations L536A and L539A, but not L540A, similarly increased mobility of the Ral-bound receptor. These results correlate well with the gains in receptor solubility in HSB extracts and of transcriptional activity of all mutants in the presence of ICI182,780, as well as of mutants L536A and L539A, but not L540A, with Ral. Together, these observations suggest that activity of the receptor in the presence of these antiestrogens is limited by its stable association with a nuclear component that results in poor extractability in HSB.

DISCUSSION

Our goals in this study were to analyze the molecular basis of the more complete transcriptional repression of ERα by the antiestrogens Ral and ICI182,780 than by OHT in cell line models. Our results show that despite the similar structures of the receptor LBD in the presence of OHT and Ral (57, 58), Ral, like ICI182,780, leads to a marked reduction in the levels of ERα extractable in HSB in HepG2 cells. These observations are not specific to HepG2 cells, as receptor levels in the presence of Ral were also intermediate between those observed with OHT and full antiestrogens in transfected HeLa and human embryonic kidney HEK293 cells (data not shown) or in native MCF7 cells. In HepG2 cells, the observed reduction in receptor levels in HSB extracts in the presence of Ral or ICI182,780 did not correspond to a proteasome-mediated degradation of ERα as described for full antiestrogen ICI182,780 in MCF7 cells (47–49), but to insolubility in HSB. In MCF7 cells, accumulation of the endogenous receptor in an insoluble fraction in the presence of ICI182,780 was also observed in the presence of the proteasome inhibitor MG132, consistent with previous observations (49). In addition, the wt receptor was insoluble in HSB extracts when stably expressed in MDA-MB-231 cells at levels similar to those of the endogenous receptor in MCF7 cells, dem-

onstrating that receptor insolubility is not due to expression of large amounts of receptor.

Previous reports have described accumulation of ERα in aggregates in the cytoplasm of transfected COS cells (45, 50) and suggested that this was due to lack of nuclear import of the receptor (50). However, ERα remained nuclear in the vast majority of HepG2 cells in the presence of ICI182,780 or Ral. In addition, a large fraction of the receptor was immobile in the presence of Ral or ICI182,780 in FRAP experiments in HepG2 cells, contrasting with the near total mobility of unliganded receptors. Thus, it appears that not only degradation but also tighter association with a nuclear compartment can account for the reduced levels of receptor in HSB extracts in the presence of full antiestrogens or Ral.

Taken together, these observations suggest that the dramatically reduced transcriptional activity of ERα in the presence of Ral *vs.* OHT in HepG2 cells results from much lower levels of functional nuclear receptors even in the absence of degradation or relocalization to the cytosol. In support of this hypothesis, our results indicate that all H12 mutants with increased agonist activity in the presence of Ral had increased solubility in HSB in HepG2 cells. The same correlation was established in the presence of ICI182,780. Similar results were obtained in transiently transfected HeLa cells, although transcriptional activity in the presence of all antiestrogens was weaker in HeLa cells (data not shown). Note also that gains in transcriptional activation were not limited to our synthetic reporter vector, because increased transcription of the endogenous estrogen target gene TFF1 in the presence of Ral or ICI182,780 was observed in MDA-MB-231 cells stably transfected with the L539A mutant. Together, these results suggest that the decreased concentration of high-salt extractable receptor contributes to the transcriptional inhibition observed in HepG2 cells in the presence of either Ral or of ICI182,780. The increase in the fraction of mobile receptors observed in FRAP experiments with mutant receptors that have increased solubility and transcriptional activity further suggests that immobilization of the receptor is due to tighter interaction with a nuclear component responsible for poor extraction in HSB and inactivity of the receptor.

The observation that Ral and ICI182,780 share functional properties in this experimental system may seem surprising in view of the fact that the crystal structure of Ral resembles closely that obtained with OHT, with H12 positioned in both cases in the coactivator binding groove (57, 58), whereas H12 is unresolved in the crystal structure of rat ERβ with the full antiestrogen ICI164,384 (59), a close relative of ICI182,780 (35, 36). Note, however, that H12 is also highly disordered in Ral-bound rat ERβ (77). Furthermore, crystal structures may trap H12 in one of several possible conformations, as suggested by the observation that H12 can be found in the coactivator binding groove, even in the presence of agonists in a transcriptionally active ERα LBD mutant (78), or in the rat ERβ LBD in the presence of the partial agonist genistein (77). Thus, Ral and OHT have differential effects on conformational equilibrium of H12 in solution and potentially also play different roles in the conformational stabilization of the whole ER LBD observed with agonists (79).

Lack of stable association of H12 with the LBD in the presence of antiestrogens may play an important role in accumulation of ERα in an insoluble fraction and/or in its degradation. Receptors with unstable H12 would expose hydrophobic regions to the surface of the protein, such as the hydrophobic face of H12 itself or the coactivator binding groove, which is normally protected by protein-protein interactions with coactivators or H12. While this article was in preparation, Wu *et al.* (80) published the structure of the ERα LBD complexed to the antiestrogen GW5638, which induces degradation of the receptor. This study concluded that binding of GW5638 leads to increased exposure of hydrophobic regions and that reducing surface hydrophobicity of H12 by the combined mutation of three hydrophobic amino acids (leucines 536, 539, and 540) to glutamine residues stabilized the receptor. Accumulation of the receptor in an insoluble fraction in HepG2 cells in the presence of Ral or ICI182,780 may also be caused by increased exposure of hydrophobic regions, because mutations reducing the hydrophobic character of H12 were found to restore receptor levels in the HSB fraction. Nonetheless, our results indicate that replacement of two other H12 hydrophobic residues (Y537 and L541) had little effect. This observation together with the differential effect of mutations on receptor solubility/activity in the presence of Ral *vs.* ICI182,780 demonstrates the importance of specific hydrophobic amino acids rather than of global H12 hydrophobicity.

Our modeling studies from available crystal structures indicate that the side chains of Ral and ICI164,384 create steric clashes with H12 in the agonist or antagonist position at the level of amino acids L536 (ICI164,384 with H12 in the coactivator binding groove), L540 (Ral and ICI164,384 with H12 in the agonist position and ICI164,384 with H12 in the coactivator binding groove), and M543 (ICI164,384 with H12 in the agonist position). Of interest, the capacity of mutation L540 to relieve local steric hindrance with the side chain of ICI182,780, but not Ral, correlates with gains in receptor solubility/activity only in the presence of ICI182,780. Mutation of residues not directly involved in steric clashes with the antiestrogen side chain may also increase space available for rearrangement of the antiestrogen side chain or of neighboring bulky amino acids. In addition, L544 points toward the Ral backbone in the ligand binding cavity when H12 is in the agonist position, and its mutation may allow for a better accommodation of Ral, which is bulkier than steroid derivatives or OHT in this region. Overall, replacement of long hydrophobic amino acids of H12 by alanine residues may facilitate burial of the hydropho-

bic side of H12 by reducing structural constraints due to the antiestrogen side chains in a residue- and ligand-specific manner. It is also possible, however, that specific hydrophobic amino acids of H12 mediate recognition of uncharacterized protein(s) important for accumulation of the receptor in an insoluble form.

Although increasing solubility of the receptor in the presence of ICI182,780 or Ral correlates with partial agonist activity in HepG2 cells, it is not necessarily sufficient because levels of pS2/TFF1 transcriptional activity were low in the presence of OHT with wt ERα in spite of high levels of soluble receptor. Also, complete deletion of H12 increased solubility in the presence of either ICI182,780 or Ral but, contrary to single mutations in long hydrophobic residues, did not lead to significant gains of transcriptional activity with the minimal ERE3-TATA promoter used in this study (data not shown). Similarly, the double mutation L539–540A was very weakly active under our experimental conditions, although soluble receptor levels were increased in the presence of ICI182,780 or Ral (data not shown). Activity of the latter mutant with ICI164,384 or ICI182,780 has been reported in HepG2 cells cotransfected with the glucocorticoid receptor interacting protein 1 (GRIP1) coactivator or in COS-1 cells with an ERE-tk-CAT reporter vector (60, 62), suggesting promoter and cell specificity in transcriptional activity of this mutant in the presence of antiestrogens. Transcriptional activity in the presence of antiestrogens appears thus to require additional functional determinants compared with those responsible for receptor solubility.

We found that the presence of the AF-1 region was important for partial transcriptional activity, both of the wt receptor with OHT and of stabilized mutant receptors with Ral or ICI182,780. We did not observe recruitment of LXXLL motifs in a two-hybrid assay, suggestive of an inactive AF-2 function, either with OHT-bound wt receptor or with mutants with increased activity in the presence of Ral or ICI182,780. Nevertheless, we cannot exclude that antiestrogens allow weak AF-2 activity, requiring cooperativity with AF-1 to be detected. Alternatively, the LBD surface formed in the presence of partial antiestrogens, or of full antiestrogens in permissive ERα mutants, may recruit specific coactivators through different motifs. Finally, recruitment of corepressors, leading to suppression of AF-1 activity, may be affected in a differential manner depending on effects of ligands or mutations on LBD conformation. Corepressor recruitment was found to be stronger with ICI182,780 and Ral than with OHT (43). Corepressor interaction with the LBD involves amino acids buried by H12 both in the agonist (on top of the ligand cavity) or antagonist (in the coactivator binding groove) positions (81), and thus mutations increasing association of H12 in either position would be expected to reduce corepressor recruitment.

Fluorescence anisotropy data measuring conformational flexibility in the vicinity of C530 indicate that the antiestrogen-bound L536A/L539A ERα-LBDs showed significantly higher anisotropy values ($P < 0.01$) than their corresponding wt ERα complexes, reaching values even greater than that of the ERα-E2 complex for some complexes. This may result from an increased degree of α-helicity of the end of H11 and/or from conformational stabilization of this region of the LBD. We speculate that this effect may result from facilitated positioning of H12 in a manner that decreases receptor insolubility in the presence of ICI182,780 or Ral and opens up the LBD surface for interactions with coactivators or with the AF-1 transcriptional activation function, or inhibits recruitment of corepressors in the ERα-antagonist complexes. In addition, the higher levels of bioluminescence energy transfer obtained in the presence of Ral or ICI182,780 in live HEK293 cells is compatible with a different conformation of the C-terminal end of the receptors, although it could also directly reflect a higher local concentration of receptors within multimers or aggregates. However, the similar association rates of the receptor in the presence of the antiestrogens or of E2 indicates that conformational alterations are likely local and do not affect the whole LBD.

In conclusion, results presented in this paper demonstrate that both Ral and ICI182,780 induce tighter association of the receptor with a nuclear compartment in HepG2 cells, that long hydrophobic amino acids of LBD H12 play a role in decreasing receptor mobility, extractability, and activity in an antiestrogen-specific manner, likely through differences in local conformation of the receptor LBD, without affecting some of its properties such as dimerization efficiency. Future experiments will be required to characterize the molecular interactions underlying the tighter association of the receptor with the nuclear compartment. While this article was under preparation, ICI182,780-specific interaction between ERα and cytokeratins 8 and 18 has been described, but this interaction appears to mediate receptor degradation rather than insolubility because the latter takes place in CK8-CK18-negative HeLa cells (82). Mutant receptors characterized here will be useful to identify proteins playing a role in receptor association with the nuclear compartment in the presence of Ral or ICI182,780, on the basis of their interaction with wt but not mutant receptors. Characterizing the patterns of mutant receptor association with corepressors should also clarify the respective roles of corepressor recruitment and association with a nuclear compartment in the full antagonist activity of antiestrogens.

MATERIALS AND METHODS

Plasmids and Reagents

E2 and OHT were purchased from Sigma (Sigma, Oakville, Ontario, Canada); ICI182,780 and Ral were purchased from Tocris Cookson Ltd. (Ellisville, MO) and Sigma, respectively.

Radioactive E2 ([^{3}H]E_2, 54 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NJ) and tetramethylrhodamine-5-maleimide (MTMR) from Molecular Probes (Eugene, OR). MG132 was purchased from EMD Biosciences (La Jolla, CA). pSG5-ERα and pSG5-HEG19 (ER$\alpha\Delta$AB) and pSG5-TIF2.1 were kind gifts from Professor P. Chambon (38, 83). Mutations at positions 531, 536, 537, 539, 540, 541, 543, and 544 were introduced by site-directed mutagenesis using PCR amplification of the ERα cDNA (the sequence of oligonucleotides used for mutagenesis is available upon request). Expression plasmids for ERα mutants were generated by subcloning the digested PCR fragments into the pSG5-ERα expression vector (792-bp *Hin*dIII/*Bam*HI fragment). Clones for each mutant were characterized by restriction digest and sequencing. The ΔAB/L536A mutant was generated by subcloning a 834-bp *Xba*I fragment from pSG5-L536A into the pSG5-ER$\alpha\Delta$AB expression vector. Vectors pVP16-ERα, pM-peptide α/βI, and 5$\times$ GAL4-TATA-Luc were generous gifts from Dr. D. P. McDonnell (72). Mutations L536A, L539A, L540A, L541A, M543A, and L544A were introduced in the pVP16-ERα by exchanging a 1611-bp *Not*I-*Bam*HI fragment. The pcDNA3-Hygro (ERα) and (L539A) were generated by inserting 1819-bp *Eco*RI blunted fragments derived from pSG5-ERα and pSG5-L539A, respectively. To create pCMV-ERα-rLuc and mutant derivatives (L536A, Y537A, L539A, and L540A), the coding sequences of the receptors without the stop codons were amplified by PCR from the corresponding pSG5 expression vectors. The amplified cDNA fragments were then subcloned into the *Eco*RI and *Xho*I sites of pRL-CMV-rLuc (Promega BioSciences, San Luis Obispo, CA). The same approach was used to create pCMV-ERα(WT, L536A, Y537A, L539A, and L540A)-YFP using the pGFP-N1-Topaz vector (PerkinElmer Corp., Wellesley, MA) instead of pRL.

Cell Culture

HepG2 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), MCF7 cells in α-MEM supplemented with 10% FBS, and MDA-MB-231 cells in DMEM supplemented with 5% FBS. Three days before experiments, MDA-MB-231 cells were switched to phenol red-free DMEM containing 5% charcoal-stripped serum, whereas HepG2 and MCF7 cells were switched to phenol red-free DMEM containing 10% charcoal-stripped serum.

Stable clones in MDA-MB-231 cells were selected for in the presence of hygromycin (0.25 mg/ml; Invitrogen, Burlington, Ontario, Canada) following electroporation (0.25 kV, 975 μF in a Bio-Rad Gene Pulser II apparatus) with 5 μg pCDNA3-hygro, pCDNA3-hygro-ERα, or pCDNA3-hygro-L539A together with 35 μg carrier salmon sperm DNA (Invitrogen).

Luciferase Assays

For luciferase assays, electroporation was carried out (Bio-Rad Gene Pulser II apparatus; Bio-Rad Laboratories, Hercules, CA) in HepG2 cells (0.24 kV, 950 μF) or in MDA-MB-231 cells (0.25 kV, 975 μF). Cells were plated in six-well plates (8×10^5 cells per well for HepG2 cells, 1×10^6 cells per well for MDA-MB-231 cells). Typically, DNA mixes contained 1 μg expression vector, 2 μg ERE3-TATA-Luc reporter vector, 2 μg internal control pCMV-βGal, and 35 μg carrier salmon sperm DNA (Invitrogen); in addition, 4 μg of the pSG5-TIF2.1 vector was used in experiments described in Fig. 8. E2 (25 nM), OHT (100 nM), ICI182,780 (100 nM), or Ral (100 nM) or vehicle (ethanol) were added immediately after electroporation. Cells were harvested 48 h later in lysis buffer (100 mM Tris-HCl, pH 7.9; 0.5% Nonidet P-40, 1 mM dithiothreitol). For proteasome inhibition, HepG2 cells were pretreated for 1 h with MG132 (10 μM) the day after transfection and subsequently treated with E2 (25 nM), OHT (100 nM), ICI182,780 (100 nM) or Ral (100 nM) for 6 h. Luciferase activity was measured in the presence of luciferin with a Fusion Universal Microplate Analyser (PerkinElmer, Woodbridge, Ontario, Canada) and was normalized for β-galactosidase activity, measured at 420 nm with a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA). Each transfection was carried out in triplicate and repeated at least three times. Statistical analysis was performed using Student's *t* test analysis.

Two-Hybrid Assays

HeLa cells were electroporated (0.24 kV, 950 μF in a Bio-Rad Gene Pulser II apparatus) and plated in six-well plates (8×10^5 cells per well). The DNA mix contained 1 μg of the expression vector for the Gal4-pep α/β I fusion protein, 1 μg expression vector for full-length wt ERα or mutants of H12 fused to VP16, 1 μg 5$\times$ GAL4-TATA-Luc reporter, 1 μg internal control plasmid CMV-β-gal, and 36 μg carrier salmon sperm DNA (Invitrogen). E2 (25 nM), OHT (100 nM), ICI182,780 (100 nM) or Ral (100 nM) or vehicle (ethanol) was added immediately after electroporation. Cells were harvested 48 h later in lysis buffer (100 mM Tris-HCl, pH 7.9; 0.5% Nonidet P-40; 1 mM dithiothreitol). Luciferase activity was measured and normalized for β-galactosidase activity as described above. All transfections were carried out in triplicate and performed a minimum of three times.

RT-PCR Assays of Receptor and TFF1 Expression Levels

Stable clones of MDA-MB-231 cells carrying the empty pCDNA3-hygro vector (0, clone 10A), or expressing the wt ERα (clone 22A) or mutant L539A (clone 4A) were kept in white DMEM supplemented with 5% treated-FBS for 72 h. Twenty four hours after plating (5×10^6 cells per 10-cm petri dish), cells were treated with vehicle, E2 (25 nM), OHT (100 nM), ICI182,780 (100 nM), or Ral (100 nM) for 48 h. RNA was extracted using TRIzol (GIBCO, from Invitrogen) according to the manufacturer's instructions. After quantification (Spectramax; Molecular Devices, Sunnyvale, CA), cDNAs were generated using 2 μg of RNA and the RevertAid H minus direct strand cDNA synthesis kit with M-MuLV reverse transcriptase (Fermentas; Burlington, Ontario, Canada).

Quantitative PCR

For quantitative PCR amplification of reverse transcribed mRNAs, the following specific oligonucleotides were used

TFF1: 5′-ACCATGGAGAACAAGGTGAT-3′, 3′-AAATTCA CACTCCTCTTCTG-5′;

p36B4: 5′-TGAAGTCACTGTGCCAGCCCA-3′, 3′-AGAAG GGGGAGATGTTGAGCA-5′

The reaction mix contained 250 nM of primers, 1/100th of the RT-PCR, Jump Start Taq DNA polymerase (Sigma, St. Louis, MO), 0.625$\times$ SybrGreen solution (Molecular Probes, from Invitrogen), 0.4 mM NTP, and $MgCl_2$ (4.0 mM for TFF1, 4.5 mM for p36B4). Samples were run on a Rotor-Gene Q-PCR machine (Corbett Research, Sydney, Australia). Similar results were obtained with three independent mRNA preparations.

Western Analysis of Receptor Levels

For Western blotting, HepG2 cells were transiently transfected by electroporation (5×10^6 cells) with 10 μg of pSG5 expression vectors containing wt or mutant ERα cDNAs and 30 μg carrier salmon sperm DNA (Invitrogen), and were plated in 10-cm plates. Stable clones derived from MDA-MB-231 cells (2.5×10^6 cells) or transfected HepG2 cells were treated with E2 (25 nM), OHT (100 nM), ICI182,780 (100 nM), Ral (100 nM) or vehicle overnight before protein extraction.

Cells were harvested in ice-cold PBS, and whole-cell extracts were prepared from half the cells by three freeze-thaw cycles in HSB as previously described (84). The other half of cells harvested was resuspended in Laemmli sample buffer (85) and incubated at 100 C for 5 min.

For western blotting of endogenous ERα in MCF7 cells, cells were plated in six-well plates (5×10^5 cells per well). The following day, cells were pretreated for 1 h with MG132 (10 μM) or vehicle (dimethylsulfoxide). E2 (25 nM), OHT (100 nM), ICI182,780 (100 nM), or Ral (100 nM) were then added for 16 h. Cells were harvested in ice-cold PBS, and whole-cell extracts were prepared as described for HepG2 cells or by resuspension in Laemmli sample buffer and incubation at 100 C for 5 min.

Whole-cell extracts from ERα-expressing cells were analyzed by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel (7.5% acrylamide) and transferred onto nitrocellulose. Membranes were incubated with an anti-ERα mouse monoclonal antibody (B10, kind gift from Professor P. Chambon). Complexes were revealed by enhanced chemiluminescence (PerkinElmer Corp.) as recommended by the manufacturer.

Confocal Fluorescence Microscopy and FRAP Analyses

HepG2 cells were plated on 35-mm γ-irradiated Corning Petri dishes (MaTek, Ashland, MA) at a density of 3000 cells/cm^2 in 2 ml white DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. After 2 d, cells were transfected with wt or mutant ERα and treated 24 h later with vehicle or ligands (E2, 2.5×10^{-5} M; OHT, ICI182,780, or Ral, 10^{-7} M) for an additional 16 h. Petri dishes were washed twice with PBS and fixed with PBS 3% paraformaldehyde for 15 min. After fixation, cells were permeabilized and blocked with PBS containing 0.2% BSA and 0.3% Triton X-100 for 10 min at room temperature. The antibody against ERα (B10, a kind gift from Professor Pierre Chambon, Strasbourg, France) and the Alexa Fluor 595 dye-labeled secondary antibody (Invitrogen) were diluted 1:800 and 1:2000, respectively, in PBS-0.2% BSA. Nuclei were stained for 5 min with 50 ng/ml Hoechst 33342 (Sigma) in PBS-0.2% BSA. Petri dishes were washed twice with PBS and once with water and were mounted using ProLong Gold antifade reagent (Invitrogen). Cells were visualized using a laser scanning microscopy (LSM) 510 META ^{MK4} confocal microscope (Carl Zeiss, Jena, Germany). Images were analyzed using LSM 3.2 software.

FRAP analysis was performed on HepG2 cells transiently transfected with expression vectors for wt or mutant ERα fused to YFP. Cells were treated as described above, except that a single region of interest (ROI) of about 25% of the nuclear volume was bleached using an Ar 488-nm laser at maximum power for 200 iterations. Emission corresponding to YFP fluorescence was captured at time intervals of 4 sec using a 505-nm LP (long pass) filter and a PMT (photomultiplicator) detector. LSM images were exported as 12 bit TIF files (256 × 256 pixels), and fluorescence intensities in the ROI and the whole cell were quantified by Meta Imaging Series 6.1. Data were analyzed using Prism Graph Pad (GraphPad Software, Inc., San Diego, CA). Fluorescence is represented in relative fluorescence units (RFU) where 0 is the fluorescence after photobleaching (time 0) and 1.00 is the expected fluorescence at homogeneity taking into account the total loss of fluorescence in the cell after photobleaching according to the formula

$$\mathrm{RFU} = \frac{(Z_i - Z_0)/Z_{ori}}{(C_i - C_0)/C_{ori}}$$

where Z_i is the average intensity in the ROI at time i, Z_0 the average intensity after bleaching in the ROI, Z_{ori} the intensity before bleaching in the ROI, C_i the average intensity in the whole cell, C_0 the estimated fluorescence background obtained after bleaching of the whole cell, and C_{ori} the average intensity of the whole cell before bleaching. Only cells displaying low to medium fluorescence intensities were analyzed (at least six cells were analyzed for each treatment).

Modeling

To compare the structural effects of the various mutations on the agonist and antagonist conformations of ERα, the crystal structures of ERα complexed with E2 [protein database (PDB) code 1GWR (71)], OHT [PDB code 3ERT (58)], Ral [PDB code 1ERR (57)], and ICI164,384 [PDB code 1HJ1, (59)] were first superimposed using the Lsq-man module of the O package [version 6 (86)]. Mutations were introduced in each crystal structure using the O package.

Expression, Purification, and Site-Specific MTMR Labeling of ERα-LBD Constructs

The expression of wt ER-LBDα(303–554) containing the single reactive cysteine at position 530 (*C530* having C381S and C417S mutations) and the L536A and L539A ER-LBDα mutants in the *C530* background and their site-specific labeling with MTMR were performed as described previously (76).

Fluorescence Anisotropy Experiments

Fluorescent anisotropy analysis of the different ER-LBD constructs labeled with MTMR was performed essentially as described previously (76). Briefly, a sample of 2 nM MTMR-labeled wt or L536A or L539A mutant was incubated with 100 nM of the respective unlabeled LBDs in Tris-glycerol (pH 8.0) buffer containing 0.3 mg/ml chicken ovalbumin for 5–7 h at room temperature in the dark. Excess unlabeled LBD was added to minimize homo-FRET artifacts by ensuring, after dimer equilibration, that only one member of the LBD dimer contained the fluorophore (76). A 700-μl sample was placed in separate tubes, and 5 μl of vehicle (dimethylsulfoxide) or 700 μM ligand stock was added, resulting in 5 μM final ligand concentrations. After equilibration for 1 h at room temperature in the dark, samples were individually analyzed at 25 C in a Spex Fluorolog II (model IIIc) cuvette-based fluorometer, with an L-configuration polarization unit using Data Max 2.2 software (Spex Industries, Edison, NJ). Excitation was at 541 nm, and MTMR fluorescence anisotropy was monitored at 580 nm. Results were analyzed using Prism 3.00 (GraphPad Software). Each fluorescent anisotropy value represents the mean ± SEM obtained from four independent experiments. All significant differences have $P < 0.05$ by one-way ANOVA.

Bioluminescence Resonance Energy Transfer Assays

For BRET assays, HEK293 cells were plated in 10-cm dishes (2.5 million cells per dish) and transfected with ERα-RLuc (1 μg) and ERα-YFP (6 μg) by the calcium phosphate method. Cells were washed twice in PBS 48 h later and harvested with 1.5 ml of PBS-5 mM EDTA. containing E2 (25 nM), OHT (100 nM), ICI182,780 (100 nM), or vehicle (ethanol). Aliquots containing 100,000 cells were distributed in a 96-well microplate (white Optiplate, Packard Instruments), and cells were treated with vehicle (EtOH), 25 nM E2, 100 nM OHT, 100 nM Ral, or ICI182,780 (100 nM) for 40 min at room temperature. Coelenterazine (Sigma) was added to a final concentration of 5 μM, and readings were immediately collected on a Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany), with sequential integration of signals detected at 485 nm (*Renilla* luciferase emission) and 530 nm (YFP emission). The BRET ratio was defined as described in Ref. 87. ERα-YFP to ERα-Luc ratios were calculated for each amount of transfected ERα-YFP expression vector in the presence of a fixed amount of the ERα-rLuc vector as the total YFP signal measured by direct YFP stimulation [YFP] minus the basal signal from cells

transfected with only ERα-rLuc [YFP_0] divided by the rLuc signal [rLuc] in the cotransfected cells.

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None of the authors have anything to declare.

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