Estrogen Receptor α L543A,L544A Mutation Changes Antagonists to Agonists, Correlating with the Ligand Binding Domain Dimerization Associated with DNA Binding Activity*

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A ligand-dependent nuclear transcription factor, ERα has two transactivating functional domains (AF), AF-1 and AF-2. AF-1 is localized in the N-terminal region, and AF-2 is distributed in the C-terminal ligand-binding domain (LBD) of the ERα protein. Helix 12 (H12) in the LBD is a component of the AF-2, and the configuration of H12 is ligand-inducible to an active or inactive form. We demonstrated previously that the ERα mutant (AF2ER) possessing L543A,L544A mutations in H12 disrupts AF-2 function and reverses antagonists such as fulvestrant/ICI182780 (ICI) or 4-hydroxytamoxifen (OHT) into agonists in the AF2ER knock-in mouse. Our previous in vitro studies suggested that the mode of AF2ER activation is similar to the partial agonist activity of OHT for WT-ERα. However, it is still unclear how antagonists activate ERα. To understand the molecular mechanism of antagonist reversal activity, we analyzed the correlation between the ICI-dependent estrogen-responsive element-mediated transcription activity of AF2ER and AF2ER-LBD dimerization activity. We report here that ICI-dependent AF2ER activation correlated with the activity of AF2ER-LBD homodimerization. Prevention of dimerization impaired the ICI-dependent ERE binding and transcription activity of AF2ER. The dislocation of H12 caused ICI-dependent LBD homodimerization involving the F-domain, the adjoining region of H12. Furthermore, F-domain truncation also strongly depressed the dimerization of WT-ERα-LBD with antagonists but not with E2. AF2ER activation levels with ICI, OHT, and raloxifene were parallel with the degree of AF2ER-LBD homodimerization, supporting a mechanism that antagonist-dependent LBD homodimerization involving the F-domain results in antagonist reversal activity of H12-mutated ERα.

Estrogen regulates physiological responses in target cells by means of intracellular estrogen receptors (ERs)2. Major estrogentic activity appears through the nuclear ERα and ERβ that activate target genes related to biological tissue responses directly as ligand-dependent transcription factors (1, 2). ERs consist of homologous structural domains, designated A to F (Fig. 1), that are shared between the nuclear receptor superfamily (3, 4). ERα has two transactivating functional (AF) domains, AF-1 and AF-2. AF-1 is localized in the A/B domains, and AF-2 is distributed in the E-domain or ligand-binding domain (LBD) of the ERα protein. Helix 12 (H12) of ERα located in the LBD is a component of the AF-2 domain. The configuration of H12 is changed by agonist or antagonist binding to the active or inactive form of ERα protein, respectively (5). ERα makes a homodimer to bind the estrogen-responsive element (ERE) within the promoter region of target genes to regulate transcription. ERα contains two dimerization signals, a ligand-inducible major dimerization function in the LBD and a constitutive weak dimerization function associated with the DNA binding domain (DBD) or C-domain (6, 7). The mutation of L511R on helix 11 (H11) of mouse ERα has been reported to result in an E2 non-active mutant because of disruption of homodimerization (6). The results of crystallography support a structure that shows H11 making an interface of ERα LBD monomers to form the dimer (5, 8).

We demonstrated previously the in vivo biological functionality of the ERα H12 mutant that possesses L543A,L544A mutations (AF2ER) (9, 10). These mutations disrupted the AF-2 function and resulted in a reversal of antagonists, such as fulvestrant/ICI182780 (ICI) and tamoxifen into agonists both in vitro in cell-based experiments and in vivo in the AF2ER knock-in (AF2ERKI) mouse. The AF2ERKI mouse expresses

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2 The abbreviations used are: ER, estrogen receptor; AF, transactivating function; LBD, ligand-binding domain; H12, helix 12; ERα, estrogen-responsive element; DBD, DNA-binding domain; ICI, fulvestrant/ICI182780; E2, estradiol; OHT, 4-hydroxytamoxifen; SERM, selective estrogen receptor modulator; S, sense; AS, antisense; TRE, Tet-responsive element; AD, activation domain; Ral, raloxifene; SUMO, small ubiquitin-like modifier.

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Antagonist-mediated ERα Activation

AF2ER mutant protein in the classical estrogen target tissues, but endogenous estradiol (E2) does not activate AF2ER. Thus, the phenotype of the AF2ERKO mouse is quite similar to the ERα knockout (αERKO) mouse that expresses no ERα protein (11), indicating that this mutant receptor is essentially inactive. However, unlike αERKO mice, ICI and TAM activate the AF2ER mutant ERs and mediate physiological responses in the AF2ERKO mice, such as uterotrophic effects in the female (9) and male reproductive tract functions (10). The mode of ICI-mediated AF2ER activation is similar to the partial agonist activity of 4-hydroxytamoxifen (OHT) on WT ERα. Namely, the overexpression of transcription coactivator, p300/cAMP-response element-binding protein (CREB)-binding protein enhanced both ICI-mediated AF2ER activation and OHT-mediated WT ERα activation through the A/B domains of ERα protein in a similar manner (9). This observation supported the previous findings, which suggest that the OHT-mediated partial agonist activity for WT ERα can only be mediated by AF-1 (12). However, there is little information available about the mechanism of antagonist-mediated ERα activation. In this report, we analyze the correlation between ICI-dependent ERE-mediated transcription activity and LBD dimerization activity of AF2ER for a further understanding of the molecular mechanisms of antagonist-mediated ERα transcription activation.

We demonstrate here that the ICI-dependent ERE-mediated transcriptional activity of AF2ER is associated with the AF2ER-LBD dimerization activity. Furthermore, prevention of dimerization impaired the ICI-dependent AF2ER activation and ERE binding of AF2ER. Additionally, we suggest that the dislocation of H12 causes ICI-dependent LBD homodimerization involving the F-domain of ERα, which facilitates dimerization. Dislocation of the ICI-dependent homodimeric LBD occurred not only with AF2ER but also with WT ERα. Understanding the mechanism of antagonist-mediated AF2ER activation will provide insights into the mechanism of partial agonist/antagonist activity of selective estrogen receptor modulators (SERMs).

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids used for reporter assay are as follows: pcDNA3-mERα; the pcDNA3 plasmid contains full-length mouse ERα (mERα1–599), pcDNA3–121-ERα; the pcDNA3 plasmid contains N-terminal 120-amino acid truncated mouse ERα (mERα121–599), pcDNA3-AF2ER; the pcDNA3 plasmid contains L543A,L544A mutated full-length mouse ERα (mERα1–599, L543A,L544A), and pcDNA3–121-AF2ER; the pcDNA3 plasmid contains L543A,L544A mutated N-terminal 120-amino acid truncated mouse ERα (mERα121–599, L543A,L544A) (9). The plasmid 3xERE-TATA-Luc (pGL3–3xERE-TATA-Int-Luc) was used for the ERE reporter gene. Additionally, we suggest that the OHT-mediated partial agonist activity for WT ERα can only be mediated by AF-1 (12). However, there is little information available about the mechanism of antagonist-mediated ERα activation. In this report, we analyze the correlation between ICI-dependent ERE-mediated transcription activity and LBD dimerization activity of AF2ER for a further understanding of the molecular mechanisms of antagonist-mediated ERα transcription activation.

The amplified fragment was cloned into pCR2.1 (Invitrogen) by the TA cloning kit (Invitrogen) and sequenced (National Institute of Environmental Health Sciences sequencing laboratory). The fragments were excised from the plasmids pcR2.1-LBD(ERαΔF)_Xhol and pcR2.1-LBD(AF2ΔF)_Xhol by Xhol and then subcloned into the Xhol sites of the pcDNA3-mERα plasmid. The direction of the inserted fragment was determined by Smal digestion. The plasmids pcDNA3-mERαΔH12, pcDNA3-mERα-L511R, and pcDNA3-AF2ER-L511R were generated by PCR-based, site-directed mutagenesis, and the following oligo DNAs were used for the mutagenesis: ΔH12_S, 5'-CCT CTA TGA TGC CCA CCG CCT TCA TGC CCC AGC-3'; ΔH12_S, 5'-GCG GTG GGC ATC ATA GAG GGG TAG CTC AGC GCC TTC TCA TTC TTG CCA GC-3'; and L511R_S, 5'-GCC GCC TAG CTC AGC GCC TTC TCA TTC TTG CCA GC-3'. PCR was performed using the Pfu Turbo DNA polymerase (Agilent Technologies), a pair of sense (S) and antisense (AS) oligo DNAs, and the plasmids pBluescript-mERα-Xhol or pBluescript-AF2ER-Xhol (the Xhol fragment from pcDNA3-mERα or pcDNA3-AF2ER was subcloned into the Xhol site of pBluescript) as a template, following the instruc-
tions of the manufacturer (Agilent Technologies). Mutated clones were confirmed by sequencing and then subcloned into the XhoI sites of pcDNA3-mERα. The direction of the inserted fragment was determined by NotI digestion. Plasmids used for mammalian two-hybrid assay are as follows: the plasmid pACT (Promega) was used for the prey, the plasmid pBIND (Promega) was used for the bait (this plasmid includes the Renilla luciferase expression cassette for internal control), and the plasmid pG5-Luc (Promega) was used for the Gal4 binding element reporter gene. The cDNAs of mouse ERα/H9251 WT and the AF2ER LBD regions were amplified by PCR using the following primer set: mE/F-5/H11032, 5/H11032-GGA TCC AGC ACA CTA AGA AGA ATA GCC CTG CCT T-3/H11032 and mE/F-3/H11032, 5/H11032-GGT ACC TGG GAG CTC TCA GAT CGT GTT GGG-3/H11032. The amplified fragment was cloned into pCR2.1 by TA cloning kit and sequenced. To generate the plasmids pACT-LBD/WT, pACT-LBD/AF2ER, pBIND-LBD/WT, and pBIND-LBD/AF2ER, LBD fragments were excised from the plasmids pCR2.1-mE/F(WT) and pCR2.1-mE/F(AF2) by BamHI and KpnI and then subcloned into the BamHI and KpnI sites of pACT or pBIND. The plasmids pACT-LBD/WTΔF, pACT-LBD/AF2ERΔF, pBIND-LBD/WTΔF, and pBIND-LBD/AF2ERΔF were generated as follows. The cDNAs of mouse ERα WT and the AF2ER LBD regions were amplified by PCR using the following primer set: mE/F-5, 5′-GGA TCC AGC ACA CTA AGA AGA ATA GCC CTG CCT T-3′ and mERαΔF-3′(KpnI), 5′-GGT ACC TAG CGA CTG GCT GGG GCA TGA-3′. The amplified fragments were cloned into pCR2.1 by TA cloning kit and sequenced. The fragments were excised from the plasmids pCR2.1-LBD(ΔF)_KpnI and pCR2.1-LBD(AF2AF)_KpnI by BamHI and KpnI and then subcloned into the BamHI and KpnI sites of pACT or pBIND. The plasmids pACT-LBD/ΔH12, pBIND-LBD/ΔH12, pACT-LBD/WT-L511R, pBIND-LBD/WT-L511R, pACT-LBD/AF2ER-L511R, and pBIND-LBD/AF2ER-L511R were generated by PCR-based, site-directed mutagenesis. The same sets of oligo DNAs as described (ΔH12_S, ΔH12_AS, L511R_S, and L511R_AS) were used for the mutagenesis. The PCR was performed using the Pfu Turbo DNA polymerase, a
pair of S and AS oligo DNAs, and the plasmids pCR2.1-mE/F(WT) or pCR2.1-mE/F(AF2) as a template. Mutated clones were confirmed by sequencing and then subcloned into the pACT or pBIND plasmids. Plasmids used for protein degradation assay are as follows. The plasmid pTet-off (Clontech) was used for the tetracycline-dependent suppression of the Tet-responsive element (TRE) containing plasmids-derived gene expression, and the plasmid pCMV-SPORT-β-gal was used for internal control. The plasmids pcTRE-mERa, pcTRE-AF2ER, pcTRE-Era(L511R), and pcTRE-AF2ER(L511R) were generated as follows. The ScaI-EcoRI fragment containing the TRE with the minimal CMV promoter was excised from the pTRE plasmid (Clontech) and the excised fragment was subcloned into the ScaI and EcoRI sites of pcDNA3-mERa, pcDNA3-AF2ER, pcDNA3-Era(L511R), and pcDNA3-AF2ER(L511R).

**Cell Culture and Transfection Conditions for the Luciferase Assay**—HepG2 (human hepatocellular carcinoma) cells were cultured in phenol red-free α-minimal essential medium supplemented with 10% FBS and 1% penicillin-streptomycin (Sigma-Aldrich). For transient transfections, the cells were cultured in phenol red-free medium supplemented with 10% charcoal-stripped FBS (Gemini-Bio) and seeded in 48-well plates at a density of 1.2 × 10⁶ cells/well. The cells were transfected with the following DNA mixture for 6 h using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. For the reporter assay, a DNA mixture containing 50 ng of expression plasmids for WT or mutated Erα (Fig. 1A), 100 ng of reporter plasmid for 3xERE-TATA-luc, and 100 ng of Renilla luciferase expression plasmid pRL-TK (Promega) was transfected in each well. For the mammalian two-hybrid assay, a DNA mixture containing 50 ng of expression plasmids for Gal4-DBD fusion proteins (pBIND), 50 ng of expression plasmids for VP16 activation domain (AD) fusion proteins (pACT) (Fig. 1B), and 100 ng of pG5-Luc reporter plasmid was transfected in each well. The pBIND plasmid contains a Renilla luciferase expression unit for transfection normalization. To analyze the coactivator motif interaction with the LBD, a DNA mixture containing 50 ng of expression plasmid for Gal4-DDB fusion SRC1 NR-box (amino acids 621–765 of human SRC1a) (pM-SRC1-NR) (13), 50 ng of expression plasmids for VP16-AD fusion proteins (pACT), 100 ng of pG5-Luc reporter plasmid, and 100 ng of pRL-TK was transfected in each well.

**Luciferase Assay**—The cells were cultured in fresh medium supplemented with E2 (Sigma-Aldrich), ICI (Tocris Biosciences), OHT (Sigma-Aldrich), or raloxifene (Ral, Tocris Biosciences) 6 h after transfections. Luciferase and Renilla luciferase activities were assayed 18 h after treatments. Luciferase activity was normalized for transfection efficiency using Renilla luciferase as an internal control. All results are representative of at least three independent experiments and represent the mean ± S.D. of triplicate samples.

**Western Blot Analysis**—Cell lysates for the protein degradation assay and DNA binding assay were prepared by the following extraction method. Cells were lysed in an extraction buffer containing 50 mM Tris/HCl (pH8.0), 5 mM EDTA, 1% Nonidet P-40, 0.2% sarkosyl, 0.4 M NaCl, 1× Halt protease inhibitor mixture (Pierce), and 1 mM dithiothreitol by vortexing, followed by a 15-min incubation on ice. After centrifugation for 5 min (21,000 × g at 4 °C), the protein amount in the supernatant was determined by BCA assay (Pierce). Proteins were resolved by SDS-PAGE and subsequently transferred to nitrocellulose membranes. Blots were incubated overnight in 4 °C with primary antibody for Erα (1:650, catalog no. MC-20, Santa Cruz Biotechnology; 1:350, catalog no. H-184, Santa Cruz Biotechnology; or 1:200, catalog no. TE111.5D11, Thermo), β-actin (1:1500, catalog no. AC-74, Sigma), or β-galactosidase (1:1000, catalog no. ab616, Abcam). We found that the F-domain of mERα is the antigenic site of MC-20. Therefore, we used H-184 (MC-20 and H-184) and TE111.5D11 to determine the F protein expression. The blots were washed and then incubated with IRDye infrared dye-conjugated anti-rabbit antibody (LI-COR Biosciences) for Erα (1:650, catalog no. MC-20, Santa Cruz Biotechnology; 1:350, catalog no. H-184, Santa Cruz Biotechnology; or 1:200, catalog no. TE111.5D11, Thermo), β-actin (1:1500, catalog no. AC-74, Sigma), or β-galactosidase (1:1000, catalog no. ab616, Abcam). We found that the F-domain of mERα is the antigenic site of MC-20. Therefore, we used H-184 and TE111.5D11 to determine the ΔF protein expression. The blots were washed and then incubated with IRDye infrared dye-conjugated anti-rabbit antibody (LI-COR Biosciences) for Erα (MC-20 and H-184) and β-galactosidase or with IRDye infrared dye-conjugated anti-mouse antibody (LI-COR Biosciences) for Erα (TE111.5D11) and β-actin. The signals were visualized with an Odyssey infrared imaging system (LI-COR Biosciences).

**Protein Degradation Assay**—HeLa cells were cultured in phenol red-free DMEM supplemented with 10% FBS and 1% pen-
icillin-streptomycin. For transient transfections, the cells were cultured in phenol red-free medium supplemented with 10% charcoal-stripped FBS and seeded in 60-mm dishes at a density of 1.0 × 10^6 cells/dish. The cells were transfected with the following DNA mixture for 12 h using Lipofectamine 2000. Cells were transfected with DNA mixture containing 0.5 μg pcTRE-mERa, pcTRE-ERa(L511R), pcTRE-AF2ER, or pcTRE-AF2ER(L511R) plasmid, 1.5 μg of pTet-off plasmid, and 0.5 μg of β-galactosidase expression plasmid pCMV-SPORT-β-gal. After 12 h, cells were treated with 1 μg/ml doxycycline (Clontech) to suppress the synthesis of WT and mutant ERα from the pcTRE plasmids. Cells were cultured with or without 100 nM ICI and harvested 2, 4, and 6 h after doxycycline treatment.

DNA Binding Assay—The DNA binding assay was performed using the NoShift transcription factor assay kit (Novagen) with the following modifications to the instructions of the manufacturer. The cell lysates that expressed WT and mutant ERα proteins were incubated on ice for 90 min in the 24-μl reaction mixture containing 1xNoShift binding buffer (Novagen), 0.005 units/μl poly(dI-dC), 25 ng/μl salmon sperm DNA, and 0.5 pmol/μl biotinylated 1xERE (S, 5′-GTC CAA AGT CAG GTC ACA GTG ACC TGA TCA AAG TT-3′, and AS, 5′-GGT CAA AGT CAG GTC ACA GTG ACC TGA TCA AAG TT-3′) to allow the binding of recombinant ERα proteins to the DNA.
Antagonist-mediated ERα Activation

**A** Two-hybrid

| E2 (nM) | pACT | LBD/WT | pACT | LBD/ERα-L511R | pACT | LBD/ΔH12 | pACT | LBD/ERαΔF |
|---------|------|--------|------|----------------|------|--------|------|--------|
| 0       | 0    | 0      | 0    | 0              | 0    | 0      | 0    | 0      |
| 0.1     | 0    | 0      | 0.1  | 0              | 0    | 0      | 0.1  | 0      |
| 1.0     | 0    | 0      | 1.0  | 0              | 0    | 0      | 1.0  | 0      |

**B** Transcription

| E2 (nM) | pcDNA3 | ERαWT | ERα-L511R | ERαΔH12 | ERαΔF |
|---------|--------|-------|-----------|---------|-------|
| 0       | 0      | 0     | 0         | 0       | 0     |
| 0.1     | 0      | 0     | 0         | 0       | 0     |
| 1.0     | 0      | 0     | 0         | 0       | 0     |

**C** Two-hybrid

| ICI (nM) | pACT | LBD/AF2ER | pACT | LBD/AF2ER-L511R | pACT | LBD/ΔH12 | pACT | LBD/AF2ERΔF |
|----------|------|-----------|------|-----------------|------|---------|------|-----------|
| 0        | 0    | 0         | 0    | 0              | 0    | 0       | 0    | 0         |
| 1.0      | 0    | 0         | 1.0  | 0              | 0    | 1.0     | 0    | 1.0      |
| 10       | 0    | 0         | 10   | 0              | 0    | 10      | 0    | 10       |

**D** Transcription

| ICI (nM) | pcDNA3 | AF2ER | AF2ER-L511R | ERαΔH12 | AF2ER-ΔF |
|----------|--------|-------|-------------|---------|---------|
| 0        | 0      | 0     | 0           | 0       | 0       |
| 1.0      | 0      | 0     | 1.0         | 0       | 0       |
| 10       | 0      | 0     | 10          | 0       | 0       |

**E**

Gal4-DBD
VP16-AD

Actin

**F**

ERα
Actin

ERα
Actin
ICI-mediated AF2ER activation was diminished by the N-terminal truncation to the WT ER activities of WT ER.

To measure the absorbance at 450 nm with a plate reader, the samples were analyzed by GraphPad Prism (GraphPad software), and two-way ANOVA with Bonferroni’s multiple comparison test was considered statistically significant.

RESULTS

The Pure ERα Antagonist Activates ERE-mediated Transcription through AF2ER—We demonstrated the antagonist reversal activity of AF2ER for ERE-mediated transcription with an *in vitro* transient transfection assay using ERα-negative HepG2 cells. As shown in Fig. 2A, 0.1 nM and higher concentrations of E2 produced activation of the ER reporter with WT ERα (Fig. 2A, ○) and a reduced activation with the N-terminal truncated ERα (121-ERα) because of the loss of AF-1 function (B, ●). The activities of WT ERαs and 121-ERα were not induced by the pure ERα-antagonist ICI (Fig. 2B, A and B, ○). In contrast, 1.0 and 10 nM ICI activated the ER-mediated transcription of AF2ER (Fig. 2C, ○) but not with E2 (●). As shown in Fig. 2D, ICI-mediated AF2ER activation was diminished by the N-terminal truncation of AF2ER (121-AF2ER). Furthermore, we analyzed ligand-dependent p160 coactivator (SRC1) recruitment to the WT ERαs and AF2ER LBDs using a mammalian two-hybrid assay. HepG2 cells were cotransfected with a Gal4-responsive reporter (pG5-Luc) and vector for the Gal4-DBD fused to the SRC1 NR-box that contains the three LXXL motifs (13) in the presence of the vectors for VP16-AD fused to the amino termini of either the WT or AF2ER LBD. Cells were treated with a series of concentrations of E2 or ICI (0.01–10 nM). The SRC1 NR-box bound to the WT LBD with 10 nM E2 (Fig. 3A, □) but not ICI (B, □). On the other hand, the SRC1-NR-box did not bind to AF2ER-LBD, neither with E2 nor ICI (Fig. 3A, B, and □). These results suggest that the AF2ER mutation has completely inactivated the AF-2 function and that AF-1 is necessary for ICI-mediated AF2ER activation.

**FIGURE 5.** LBD dimer formation and transcription activities. A, HepG2 cells were cotransfected with pG5-luc and expression vector for Gal4-DBD fused WT or mutated ERα LBDs (pBIND-LBD/WT, pBIND-LBD/ERα-L511R, pBIND-LBD/ERα-H12, or pBIND-LBD/ERα-D3Δf) in the presence of the expression vector for VP16-AD (pACT) or VP16-AD fused WT or mutated ERα LBDs (pACT-LBD/WT, pACT-LBD/ERα-L511R, pACT-LBD/H12, or pACT-LBD/D3Δf) in the presence of the expression vector for VP16-AD (pACT) or VP16-AD fused WT or mutated ERα LBDs (pACT-LBD/WT, pACT-LBD/ERα-L511R, pACT-LBD/H12, or pACT-LBD/D3Δf).

**FIGURE 6.** Disruption of dimerization inhibits the ligand-dependent DNA binding of AF2ER. To detect the ligand-dependent ER binding activities of WT and mutated ERα, a biotinylated ERE probe was incubated with either vehicle (Ligand), 1 μM E2 (+E2), or ICI (+ICI) and HeLa cell extracts that were transfected with pcDNA3 (empty), WT ERα, ERα-L511R, AF2ER, or AF2ER-L511R expression plasmids. Detection was performed as described under “Experimental Procedures.” Relative DNA binding activity was represented as fold change over the pcDNA3-transfected cell level. Results are represented as the mean ± S.D. of three independent experiments. a and b, p < 0.001 against vehicle sample (Ligand) of each receptor. A representative Western blot analysis shows the levels of ERα WT and mutants and endogenous β-actin in each extract used for the DNA binding assay (inset).
ICI-dependent AF2ER LBD Dimerization Correlates with the Transcription Activity of AF2ER—Because the dimerization of ERα is an important step for transcription activation, we evaluated the correlation between ligand-dependent LBD dimerization and transcription activities. To examine the activity of LBD homodimerization, we performed a mammalian two-hybrid assay. HepG2 cells were cotransfected with pG5-Luc and vectors for the Gal4-DBD fused to the amino termini of either WT or mutated ERα/H9251 LBD (pBIND-LBD) in the presence of the vectors for VP16-AD alone (pACT) or VP16-AD fused to the amino termini of either WT or mutated ERα/H9251 LBD (pACT-LBD). Cells were treated with a series of concentrations of E2 or ICI (0.01–10 nM). At first, we demonstrated ligand-dependent WT-LBD homodimerization. As shown in Fig. 4A, we observed a relatively high basal level of luciferase activity in the pBIND-LBD/WT and pACT-LBD/WT co-transfected cells without ligand (0 nM), and that activity was increased with 1 nM E2 (Fig. 4A, right panel, black column), suggesting that the WT-LBD makes a homodimer without ligand and that the level is increased by E2. Treatment with ICI decreased the level of the WT-LBD homodimer (Fig. 4A, right panel, white column). The combination of pBIND-LBD/WT with pACT induced luciferase activity at 10 nM E2 (Fig. 4A, left panel, black column), suggesting that pBIND-LBD/WT itself induces the transcription activity at a higher E2 concentration. Thus, using this method, we could not define whether the dimerization of WT-LBD was induced at higher E2 concentrations. On the other hand, the homodimer of AF2ER-LBD was not detected at any E2 concentrations (Fig. 4B, black column). The level of the AF2ER-LBD homodimer was increased by 10 nM ICI (Fig. 4B, right panel, white column), suggesting that the ICI-dependent AF2ER-LBD homodimerization coincides with the transcription activity of AF2ER. For the following two-hybrid and reporter assays, we used concentrations of E2 from 0–1 nM for WT ERα/H9251 and concentrations of ICI from 0–10 nM for AF2ER.

Homodimerization Is Necessary for the Antagonist Reversal Activity of AF2ER—Mutation of leucine 511 to arginine (L511R) has been shown previously to inhibit the E2-dependent dimerization of mouse ERα (6). Thus, we generated the L511R-mutated ERα (ERα-L511R), AF2ER (AF2ER-L511R), and two-hybrid constructs of the LBD (LBD/ERα-L511R, LBD/AF2ER-L511R) to evaluate the significance of LBD dimer formation on the antagonist reversal activity of AF2ER. We found no luciferase activities from the pBIND-LBD/ERα-L511R with pACT-LBD/ERα-L511R- or pBIND-LBD/AF2ER-L511R with pACT-LBD/AF2ER-L511R-transfected cells treated with E2 or ICI, respectively (Fig. 5, A and C), suggesting that the L511R-mutated LBDs did not induce ligand-dependent homodimeriza-
tion. The ligand-dependent ERE-mediated transcription activations of ER\textsubscript{H9251/L511R} and AF2ER-L511R were attenuated (Fig. 5, B and D), suggesting that homodimerization is required for the ligand-dependent ER\textsubscript{A} activation and the antagonist reversal activity of AF2ER.

**Disruption of Helix 12 Causes the Antagonist Reversal Activity**—To evaluate the impact of the disruption of H12 on antagonist reversal activity, the entire H12 (seven amino acids, DLLLEML) was deleted from ER\textsubscript{H9251} (ER\textsubscript{H9004H12}), and ER\textsubscript{H9251/H9004H12} and LBD/ER\textsubscript{H9004H12} were generated. Surprisingly, we found that 10 nM ICI induced the luciferase activity in the pBIND-LBD/ER\textsubscript{H9004H12} and pACT-LBD/ER\textsubscript{H9004H12} co-transfected cells (Fig. 5C) but not E2 (A). The E2-dependent transcription activity of ER\textsubscript{H9251/H9004H12} was diminished (Fig. 5B). In contrast, ER\textsubscript{H9004H12} was activated by ICI, and that level was lower than AF2ER (Fig. 5D), correlating with the level of ICI-dependent LBD/ER\textsubscript{H9004H12} homodimerization. These results may suggest that the H12 is disoriented in the AF2ER mutant, causing the antagonist reversal activity of AF2ER.

**The F-domain Is Important for Antagonist Reversal Activity of AF2ER**—The results from the H12 mutation evoked the question of whether the disposition of H12 affects the function of the adjoining F-domain. We generated an F-domain-truncated ER\textsubscript{A} (ER\textsubscript{A}F), AF2ER (AF2ER\textsubscript{A}F), and two-hybrid constructs of LBD (LBD/ER\textsubscript{A}F, LBD/AF2ER\textsubscript{A}F) to evaluate the involvement of the F-domain in the antagonist reversal activity. In the mammalian two-hybrid assay, the basal level of luciferase activity in the LBD/ER\textsubscript{A}F-transfected cells was lower than the level of LBD/WT-transfected cells without ligand (0 nM), but it was increased with 1 nM E2 (Fig. 5A). The E2-dependent transcription activities of ER\textsubscript{H9251/H9004H12} and WT were increased in parallel with the levels of LBD/ER\textsubscript{A}F and LBD/AF2ER\textsubscript{A}F (Fig. 5B). On the other hand, the level of luciferase activity in the ICI-treated pBIND-LBD/AF2ER\textsubscript{A}F with pACT-LBD/AF2ER\textsubscript{A}F-transfected cells was impaired but was detectable at 10 nM ICI (Fig. 5C). ICI-dependent AF2ER\textsubscript{A}F transcription was attenuated and not induced in a dose-dependent manner (Fig. 5D). These results suggest that the F-domain plays a role in the antagonist reversal activity of AF2ER.

**Homodimerization Affects the Ligand-dependent DNA Binding Activity**—To evaluate the effect of homodimerization on DNA binding activity, we analyzed the ligand-dependent DNA binding activities of WT ER\textsubscript{A} and AF2ER, and L511R mutants.

**FIGURE 8. The effect of SERMs on AF2ER, AF2ER\textsubscript{A}F, AF2ER-L511R, and ER\textsubscript{A}H12 activities.** A, HepG2 cells were cotransfected with 3xERE-TATA-luc, pRL-TK, and the expression vector for AF2ER, AF2ER\textsubscript{A}F, AF2ER-L511R, H12, or pcDNA3 and then treated with either vehicle (Veh), 10 nM E2, ICI, OHT, or Veh. The luciferase activity is represented as fold change against the empty expression vector (pcDNA3) in the presence of each ligand. Luciferase activity is represented as mean ± S.D. a, b, and c, p < 0.001 against the vehicle level of each receptor. B, HepG2 cells were cotransfected with pG5-luc and the following combinations: pBIND-LBD/AF2ER in the presence of pACT or pACT-LBD/AF2ER, pBIND-LBD/AF2ER\textsubscript{A}F in the presence of pACT or pACT-LBD/AF2ER-L511R, and pBIND-LBD/H12 in the presence of pACT or pACT-LBD/H12. Cells were treated with either vehicle, 10 nM E2, ICI, OHT, or Veh. The luciferase activity is represented as a fold change over vehicle in each pACT and pBIND-LBD co-transfected sample. Luciferase activity is represented as mean ± S.D. a, c, and e, p < 0.001 against vehicle in each pACT and pBIND-LBD co-transfected sample; b, d, and f, p < 0.001 against vehicle in each pACT-LBD and pBIND-LBD co-transfected sample;
As shown in Fig. 6, the DNA binding level of WT was increased significantly by treatment with E2 or ICI compared with no-ligand (-Ligand). The DNA binding level of AF2ER was increased significantly by ICI treatment but not E2 compared with no ligand. These responses were eliminated in the ER/H9251-L511R or AF2ER-L511R mutants. These results suggest that ICI-dependent AF2ER homodimerization influences ERE binding activity.

Dimerization Does Not Affect ICI-mediated ERα Protein Degradation—We analyzed the effect of ICI on AF2ER protein stability because it is well known that ICI induces the degradation of ERα/H9251 protein (14, 15). To exclude the possibility of any chemical effect on the expression level of transfected cDNAs, we employed the tetracycline-dependent transcription repression method (Tet-Off system) for determining the protein degradation. As shown in Fig. 7, ICI induced the degradation of WT protein. On the contrary, ICI did not induce AF2ER protein degradation (Fig. 7D). To evaluate the effect of dimerization on ERα protein degradation, we analyzed the effect of ICI on ERα-L511R and AF2ER-L511R mutants. The profiles of ERα-L511R and AF2ER-L511R mutants were identical to WT and AF2ER, respectively (Fig. 7, C and E), suggesting that dimerization does not affect ICI-dependent ERα protein degradation.

The Effect of LBD Homodimerization Correlates to Antagonist Reversal Activity of AF2ER—We analyzed the effect of other types of antagonists, OHT and Ral, on the transcription and LBD homodimerization activities of AF2ER, AF2ERΔF, AF2ER-L511R, ERαΔH12 (Fig. 8), WT ERα, ERαΔF, and ERα-L511R (Fig. 9) using an ERE reporter assay and a mammalian two-hybrid assay, respectively. AF2ER and ERαΔH12-mediated transcription were activated by OHT to a lower level than ICI but were not activated by Ral (Fig. 8A). The levels of ligand-dependent LBD/AF2ER and LBD/ERαΔF homodimerization were parallel to the transcription activities of AF2ER and ERαΔH12, respectively (Fig. 8B). On the other hand, OHT and Ral induced WT-LBD homodimerization more than E2 (Fig. 8B). However, the transcription activity of WT ERα was in opposition to the level of WT-LBD homodimerization (Fig. 9A). Furthermore, F-domain truncation from WT-LBD (LBD/ERαΔF) dramatically depressed the OHT and Ral-dependent homodimerization of LBD/ERαΔF (Fig. 9B).
DISCUSSION

ICI is a pure antagonist for WT ERα and blocks transcription activity (16). A portion of the antagonist effect of ICI results from the stimulation of ERα proteolysis occurring in vitro (Fig. 7B and Ref. 14) and in vivo (15). We found that the AF2ER mutations prevented ICI-mediated ERα protein degradation (Fig. 7D). This may be a partial explanation for ICI-mediated AF2ER activation. Meanwhile, ICI reduces WT-LBD dimerization and induces AF2ER-LBD dimerization (Fig. 4). However, dimerization did not have any impact on ICI-mediated ERα proteolysis (Fig. 7, C and E). Tamrazi et al. (17) reported that ERα ligands, including ICI, stabilize the WT-LBD homodimer compared with no ligand using an in vitro FRET-based assay. The differences between their in vitro results and our findings in the mammalian two-hybrid assay suggest that the ICI-bound WT ERα may introduce cellular factors to prevent homodimerization independently of proteolysis. It has been reported that the inhibition of ERα dimerization reduces the binding activity to the consensus ERE in an EMSA (6, 18). However, the ligand dependence of ERα binding has not been clearly defined using the EMSA method. We employed a colorimetric immunoassay in which the complex of biotinylated ERE and ERα on a streptavidin-coated plate is detected by an ERα-specific antibody. These data suggest that the ERE-binding activity of AF2ER is increased by ICI but not E2 and that activity was prevented by the disruption of dimer formation with the L511R mutation (Fig. 6). The DNA binding activity of WT was increased by E2 and also ICI, and that activity was diminished by the L511R mutation the same as with the AF2ER (Fig. 6). This result would support that ICI stabilizes the homodimerization of WT ERα in vitro, as Tamrazi et al. (17), and it may cause the induction of DNA binding activity with ICI in our in vitro DNA binding assay. Recently, Hilmi et al. (19) reported that ICI induces SUMOylation of human ERα protein in the cells and that the mutations of H12 (L539A or L540A, which correspond to mouse L543A and L544A) strongly reduced SUMOylation in the presence of ICI. We observed that ICI reduced the level of ERα WT-LBD homodimerization in the two-hybrid assay (Fig. 4). Therefore, ICI-mediated, posttranslational modification of WT ERα may cause the prevention of ERα dimer formation in vivo. Taken together, these results suggest that the prevention of ICI-dependent proteolysis maintains the dimer formation of AF2ER and that this increases ERE binding and transcription activation.

Previous studies have also reported that the H12 mutation in the ERα LBD changes antagonists into agonists (20–22). The L543A/L544A and M547A/L548A mouse ERα mutants exhibit reduced basal transcription activity and have lost the ability to respond to E2, but these mutants are activated by anti-estrogens, IC164384, and OHT (20). These hydrophobic amino acids (Leu-543, Leu-544, Met-547, and Leu-548) are localized on the same surface of H12 (23). The Leu-544 of mouse ERα correlates to Leu-540 in the human ERα. Montano et al. (21) have reported that the characteristics of the L540Q human ERα mutant are quite similar to the L543A/L544A mouse ERα (AF2ER) mutant. Recently, H12 mutations (M543V and M543A/L544A) in human breast cancers were reported, and both mutants were activated by ICI and OHT more than by E2 (24). The position of Met-543 and Leu-544 of human ERα correlates with the Met-547 and Leu-548 of mouse ERα that were reported by Mahfoudi et al. (20). These results suggest that the disruption of the nonpolar surface of H12 provokes antagonist reversal activity, and the possibility of tamoxifen resistance in breast cancer might arise from such a mechanism.

Here we suggest that ICI-mediated antagonist reversal activity can also be induced by the removal of the entire H12 and that the subsequent F-domain is involved in this activity (Fig. 5). Interestingly, OHT and Ral induced the dimer formation of WT-LBD more potently than E2, and F-domain truncation strongly depressed the ability of OHT/Ral-mediated WT-LBD dimerization (Fig. 9B). These results would indicate that the F-domain is closely associated with the antagonist-dependent ERα LBD dimerization. Because the F-domain is directly adjacent to H12, the location of the F-domain would be influenced by H12. Crystallographic analyses have shown that SERMs generate a differential orientation of H12, resulting in partial agonist/antagonist activity of SERMs (25, 26). Our current findings provide further consideration for the orientation of the F-domain toward understanding the precise activity of SERMs.

The crystallographic analysis indicated that the possible H12 position would not allow for a secure structure with ICI bound to the ER LBD (27). Because the H12-mutated ERα (AF2ER) is activated by ICI, we questioned whether the AF2ER-LBD could recruit p160 coactivators in a similar manner as the WT-LBD. However, the AF2ER-LBD did not recruit the SRC1-NR-box with ICI, nor with E2 (Fig. 3). This result is consistent with the fact that the N-terminal-truncated AF2ER (121-AF2ER) does not have any transcriptional activity with ligands (Fig. 2). Our earlier results suggest that the SRC-1 accelerates the p300/CBP-dependent AF2ER activation through the N-terminal transcriptional activation function (9). Our current view is that the AF2ER-LBD alone does not possess functional transcription activity and that the N-terminal (AB domains)-derived transcription activity is mediated by ligand-dependent AF2ER activation. Although Métivier et al. (28) indicated that a physical interaction between the A-domain and the LBD of ERα causes the repression of unliganded ERα transcription activity, it is still controversial how AF-1 activity is controlled in the unliganded or antagonist-bound, non-active WT ERα. Further examination will be necessary with the possibility that antagonist-induced dimerization of AF2ER-LBD may release the A-domain from the LBD to activate AF2ER transcriptional activity.

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