Molecular Basis for the Constitutive Activity of Estrogen-related Receptor α-1*

Some orphan nuclear receptors, including estrogen-related receptor α-1 (ERRα-1), can activate gene transcription in a constitutive manner. Little is known about the molecular basis of the constitutive activity of these receptors. Our results from site-directed mutagenesis experiments have revealed that Phe-329 (analogous to Ala-350 in estrogen receptor α (ERα)) is responsible for the constitutive activity of ERRα-1. The ERRα-1 mutant F329A lost the transactivation activity and acted as a dominant negative mutant. The mammalian cell transfection experiments revealed that the ERRα-1 mutant F329A, like wild-type ERα, recognized toxaphene (an organochlorine pesticide) as an agonist. This compound was previously shown to be an antagonist of wild-type ERRα-1. On the other hand, like wild-type ERRα-1, the ERα mutant A350F was found to be constitutively active (as demonstrated by mammalian cell transfection and yeast two-hybrid assays). These results indicate that Phe-329 in ERRα-1 and Ala-350 in ERα play important roles in both ligand binding and transactivation function.

Based on ligand binding properties, nuclear receptors can be classified into three types. The first type of nuclear receptor, including estrogen receptor (ER) and androgen receptor, is activated by specific ligands. The second type of receptor, similar to steroid and xenobiotic receptor, has a wide selectivity for ligands. The third type of receptor, like ERRα-1, is transcriptionally active in the absence of exogenous ligand. The third type of receptor has similar structural domain arrangements to the first and the second types of receptors. Little is known as to why receptors such as ERRα-1 are constitutively active. Our site-directed mutagenesis study has provided a molecular basis for the constitutive activity of ERRα-1.

The cDNA for ERRα-1 was first isolated by screening cDNA libraries using probes corresponding to the DNA-binding domain of the human ERα. Sequence alignment of ERRα-1, ERα, and ERβ reveals a strong similarity. In the ligand binding region, the amino acid sequence of ERRα-1 shows 36% identity when compared with ERα and 34% identity when compared with ERβ. However, ERRα-1 does not bind to any of the major classes of steroids, including estrogens and androgens (1).

Vanacker et al. (2) suggested that ligands for ERRα-1 might be present in fetal calf serum, based on results that showed a lack of the ERRα-1 transactivation activity in ROS 17/2.8 cells under stripped serum conditions. Despite efforts from several laboratories, the physiological ligand for ERRα-1 has not yet been identified. Experimentally, ERRα-1 is transcriptionally active in the absence of exogenous hormone (see Fig. 2), and therefore, this receptor is generally considered to be a constitutive transcriptional activator protein. Utilizing yeast-based assays and mammalian transient transfection assays, we have recently found that two organochlorine pesticides, toxaphene and chlordane, can act as antagonists of ERRα-1 that suppress the constitutive activity of ERRα-1 (3). These findings are similar to those for the interaction of androstane metabolites with nuclear receptor CAR-β (4). In contrast to the antagonistic action on ERα-1, these pesticides have been reported to be weak agonists of ERα (5).

EXPERIMENTAL PROCEDURES

Materials—DNA sequencing kits were from United States Biochemical (Cleveland, Ohio). T4 kinase, T4 DNA ligase, and restriction endonucleases were purchased from New England Biolabs (Beverly, MA) and Roche Molecular Biochemicals. AmpliTaq polymerase was obtained from PerkinElmer Life Sciences. [3-14C]Chloramphenicol ([D-3,4,5-14C]) chloramphenicol (specific radioactivity, 55 mCi/mmol) was from Amersham Pharmacia Biotech. The chloramphenicol acetyl transferase (CAT) expression vector, pUMVOCAT, was a gift from Dr. K. Kurachi at the University of Michigan, Ann Arbor, Michigan. Oligonucleotide primers were synthesized in the DNA/RNA chemistry laboratory at the City of Hope. SK-BR-3 cells (atCC (Manassas, VA), derived from a human breast adenocarcinoma, were maintained in McCoy's 5A medium containing 10% fetal calf serum and glutamine. Estradiol and tamoxifen were purchased from Sigma. Toxaphene was kindly provided by Dr. Michael D. Shelby at the National Institute of Environmental Health Sciences. Dimethyl sulfoxide was from Mallinkrodt Chemical Works. The pS5G-GRIP1 was kindly provided by Dr. Michael R. Stallcup (University of Southern California, Los Angeles, CA). TheERE-TK-CAT plasmid was kindly provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX).

Mutant Preparation—The mutants were generated by using a polymerase chain reaction (PCR)-based mutagenesis method described by Nelson and Long (6). Briefly, the PCR mutagenesis for the ERα-1 mutant F329A used four primers: mutant Primer A for hERRα-1/F329A, TGTGACCCTCGCTGACGAG (a forward primer with the mutated bases underlined); Primer B, GGGGTACTAGTACCCGCGGTTCTCA- GTCCATCATGGCCTCGAG (a reverse hybrid primer containing a RI restriction site, a 3′-terminal 24-nucleotide sequence complementary to the ERRα-1 cDNA in the reverse strand that encodes for the carboxyl terminus, and a 5′-terminal 20-nucleotide unique sequence); Primer C, GACGGATCCGGATATGCGTACGCAAGAG (a forward primer containing an EcoRI restriction site and the cDNA sequence containing the first ATG site at the 5′-end); and Primer D, GGGGTACTAGTACCCGGG (a primer with a sequence that is identical to the 5′-terminal 20-nucleotide sequence of Primer B). PCR was carried out with a DNA Thermal Cycler 480 (PerkinElmer Life Sciences). For step 1, the reaction mixture (in 100 µl) contained 2.5

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The abbreviations used are: ER, estrogen receptor; h, human; E2, 17β-estradiol; ERRα-1, estrogen-related receptor α-1; GRIP1, glucocorticoid receptor-interacting protein 1; PCR, polymerase chain reaction; CAT, chloramphenicol acetyl transferase; ERE, estrogen response element.
units of Taq polymerase, 1 pmol of pSG5-hERRα-1 as template, and 100 pmol each of Primers A and B. The PCR cycles were 2 min at 95 °C to denature the template DNA, 2 min at 45 °C to allow the primers to anneal, and 2 min at 72 °C for DNA extension and cycled 30 times. The PCR product was resolved over 1% agarose gel and extracted by using the Qiagen Gel Extraction Kit (Qiagen Inc., Chatsworth, CA). The Step 1 product and 1 pmol of the Step 1 product and 1 pmol of pSG5-hERRα-1 and was run as a single cycle of 5 min at 95 °C, 2 min at 45 °C, and 10 min at 72 °C. Primers C and D were then added (100 pmol of each), and 30 additional PCR cycles were completed. The final PCR product was digested with EcoRI and BamHI. The resulting full-length mutant cDNA for hERRα-1 was ligated into the pSG5 expression vector. The sequence of the pSG5-F329A construct was confirmed by sequencing.

The ERα mutant A350F was also prepared by the PCR-based mutagenesis approach. Plasmid pIC-ERF (purchased from ATCC), which contains the complete coding sequence of human ERα, was used as template. The sequences of Primers A(ER), B(ER), C(ER), and D(ER) to generate the full-length human ERα cDNA encoding hERα/A350F are: 5’-CTGACACACCCTGTCGACAGGGAG-3’, 5’-GGGTTACTAGTAACCCGGGC-3’, 5’-GCCGAATTCGGGCGGATCCTCAGACTGTGGCAGGGAAACCCTC-3’, 5’-GCCGATTCGCGCCGGCCAATGACCATGACCCTCCACACCAAAAGC-3’, and 5’-GGGTTACTAGTAACCCGGGC-3’, respectively. The PCR-amplified A350F-containing ERα cDNA fragment was purified with EcoRI and BamHI and subcloned into pSG5 vector through EcoRI and BamHI sites. The expression plasmid, pSG5-hERα, for the expression of wild-type human ERα, was generated as follows. The full-length coding region of human ERα was generated by PCR using Primers C(ER) and B(ER) (the same as those used in ER/A350F preparation) on template DNA, pIC-ERF. The PCR product was digested with EcoRI and BamHI and subcloned into pSG5 vector through EcoRI and BamHI sites.

**Construction of Yeast Expression Plasmids**—The yeast expression plasmids pGBT9-hERα/HBDWT and pGBT9-hERα/HBD350F for the expression of Gal4DBD/hERα/HBDWT and Gal4DBD/hERα/HBD350F fusion proteins were constructed as follows. The coding regions for wild-type and A350F mutant containing human ERα hormone-binding domains (amino acids 279–595) were amplified by PCR using pSG5-hERα and pSG5-A350F as template DNA, respectively. The sequence of the forward primer is 5’-CCGGAATTCATGGAAGTGGGGTCTGCTGG-3’, and the reverse primer is Primer B(ER), the same as the one used for the PCR-based mutagenesis approach.

**Fig. 1.** Comparison of amino acid residues situated in the ligand-binding sites of ERα and ERRα-1. The residues of ERα are underlined, and the residues of ERRα-1 are in italic. The residues that are homologous are in bold.

**Fig. 2.** Transactivation analysis of ERRα-1 mutant F329A. SK-BR-3 cells were transfected with a CAT reporter plasmid (pums-64/+129CAT, 4 µg) and pSG5-F329A at the indicated amounts. The cells in sets 6 to 8 were also transfected with pSG5-hERRα-1 (2 µg). CAT assay was performed 72 h after transfection. Results are expressed as relative reporter activity from two independent experiments with each concentration in triplicate. WT, wild type.

**Fig. 3.** Analysis of the interaction of wild-type ERRα-1 and the mutant F329A with GRIP1. In addition to pums-64/+129CAT (2 µg) and pSG5-GRIP1 expression plasmid (1 µg), SK-BR-3 cells were transfected with pSG5-hERRα-1 (1 µg) or pSG5-F329A (1 µg) as indicated. The CAT activity was measured after a 48-h transfection and plotted as the percentage of CAT activity from two independent experiments with each concentration in triplicate. As controls, the reporter activity in cells was transfected with the empty vector.

**Fig. 4.** Toxaphene is an agonist for F329A. SK-BR-3 cells were transfected with pums-64/+129CAT (2 µg), pSG5-hERRα-1 (2 µg), or pSG5-F329A (2 µg). The transfected cells were incubated with toxaphene for 48 h at the indicated concentrations. After cells were washed twice with 1 × phosphate-buffered saline, the CAT activity was measured. The graph represents an average of three independent experiments. WT, wild type.

**Table 1.** Comparison of amino acid residues situated in the ligand-binding sites of ERα and ERRα-1. The residues of ERα are underlined, and the residues of ERRα-1 are in italic. The residues that are homologous are in bold.

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**Table 3.** Analysis of the interaction of wild-type ERRα-1 and the mutant F329A with GRIP1. In addition to pums-64/+129CAT (2 µg) and pSG5-GRIP1 expression plasmid (1 µg), SK-BR-3 cells were transfected with pSG5-hERRα-1 (1 µg) or pSG5-F329A (1 µg) as indicated. The CAT activity was measured after a 48-h transfection and plotted as the percentage of CAT activity from two independent experiments with each concentration in triplicate. As controls, the reporter activity in cells was transfected with the empty vector.
used for A350F preparation. The PCR products were digested with a luciferase reporter plasmid (ERE/3SV40 Luc, 0.5 μg) and pSG5-A350F (0.1 μg). The transfected cells were incubated with Me2SO (DMSO) or 100 nM E2 in Me2SO for 48 h. After cells were washed twice with 1 × phosphate-buffered saline, the luciferase activity was measured. For experiments that involved GRIP1, the cells were also transfected with pSG5-GRIP1 (1 μg). B demonstrates that wild-type ERRα interacts with GRIP1 only in the presence of E2. The cells were transfected with ERE/3SV40_Luc (0.5 μg), pSG5-ERRα (0.1 μg), and when indicated, pSG5-GRIP1 (1 μg). After incubation with Me2SO or 100 nM E2 for 48 h, the reporter activities were measured.

![Graph A](image1.png)

**FIG. 5.** A, transactivation analysis of ERα mutant A350F. HeLa cells were transfected with a luciferase reporter plasmid ((ERE)3SV40 Luc, 0.5 μg) and pSG5-A350F (0.1 μg). The transfected cells were incubated with Me2SO (DMSO) or 100 nM E2 in Me2SO for 48 h. After cells were washed twice with 1 × phosphate-buffered saline, the luciferase activity was measured. For experiments that involved GRIP1, the cells were also transfected with pSG5-GRIP1 (1 μg). B demonstrates that wild-type ERRα interacts with GRIP1 only in the presence of E2. The cells were transfected with ERE/3SV40_Luc (0.5 μg), pSG5-ERRα (0.1 μg), and when indicated, pSG5-GRIP1 (1 μg). After incubation with Me2SO or 100 nM E2 for 48 h, the reporter activities were measured.

![Graph B](image2.png)

**FIG. 6.** A, transactivation analysis of ERα mutant A350F in SK-BR-3 cells. B demonstrates that wild-type ERα interacts with GRIP1 only in the presence of E2. The experimental conditions are identical to those described in Fig. 5. DMSO, Me2SO.

Mutagenesis of ERRα-1

By guest on July 24, 2018

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two washes with 1 × phosphate-buffered saline, and the CAT activity was measured.

**ER Functional Assay**—Transactivation analysis of wild-type ERα and its mutant A350F was performed using HeLa cells and SK-BR-3 cells as the host cells. The cells were transfected with a luciferase reporter plasmid pGL3/ERE<sub>μ</sub>-luciferase (0.25 μg) along with 10 μg of pSG5-hERα or pSG5-A350F using Lipofectin in Opti-MEM medium. Five h after exposure to Lipofectin/DNA, the Lipofectin/DNA-containing medium was removed, and the cells were cultured in 5% medium. Twenty-four h after transfection, the cells were harvested, lysed, and subjected to protein assay. An aliquot of cell lysates containing the same amount of protein from all the samples was used for luciferase assay according to the manufacturer's instructions (Promega). The luciferase activities were expressed as the mean and standard deviation of three independent experiments.

**Yeast Two-hybrid Assay**—The interaction between the wild-type ERα hormone-binding domain or its mutant A350F with GRIP1 was demonstrated by the yeast two-hybrid assays. The yeast strain Y187 was co-transformed with pGBT9-hERα/HBD<sub>WT</sub> or pGBT9-hERα/HBD<sub>A350F</sub> and pGAD-GRIP1<sub>322-1121</sub>. The reporter β-galactosidase activities in transformants were measured following the published procedure (7).

**Computer Modeling**—Homology models (9) were built using software marketed by Molecular Simulations Inc. (San Diego, CA). The agonist-bound form of ERRα-1 was modeled using the DES-bound form of the estrogen receptor as a template (Protein Data Bank 3ERD, Ref. 10). The antagonist form was modeled using the tamoxifen-bound form (3ERT).

## RESULTS AND DISCUSSION

We reviewed the amino acid sequence alignment between ERRα-1 and ERα (1) and compared the corresponding residues in ERRα-1 with those in the ERα ligand-binding site that are in direct contact with E2, as shown by Tanenbaum et al. (11) (Fig. 1). We have found that 9 out of the 19 residues are identical. Seven residues are conservative changes. One critical difference is between Phe-329 in ERRα-1 and Ala-350 in ERα (Fig. 1). It was thought that this difference might be the cause of the differences in ligand binding properties between the two receptors.

The phenyl group of Phe-329 in ERRα-1 could provide steric hindrance to prevent the binding of E2 to this receptor. To test our hypothesis, we prepared ERRα-1 mutants in which Phe-329 was replaced by the residues in ERα, i.e. F329A as well as the ERα mutant A350F.

Studies from our laboratory have revealed that ERRα-1 up-regulates aromatase expression in human breast tissue by binding to a regulatory element, S1, that is situated near promoters I.3 and II of the human aromatase gene (12). Aromatase is an enzyme that converts androgens to estrogens. The DNA mobility shift assay revealed that the levels of the protein-S1 complexes for the mutant F329A and wild-type ERRα-1 were very similar (results not shown), supporting our prediction that Phe-329 is situated in the ligand-binding domain, not in the DNA-binding domain.

To address the biological significance of the ERRα-1 mutation, we co-transfected the SK-BR-3 human breast cancer cells with the expression plasmid for ERRα-1 (or its mutant) along with a CAT reporter plasmid containing the aromatase genomic fragment having promoter I.3 and the ERRα-1 binding element S1 (i.e. pums-64+/129CAT, as described in Ref. 12). Although wild-type ERRα-1 enhanced promoter I.3-driven CAT activity as reported previously (12), the mutant F329A reduced the basic promoter activity (Fig. 2). Furthermore, F329A suppressed the wild-type ERRα-1 activity when they were co-expressed. These results indicate that F329A, acting as a dominant negative mutant, inhibited the activity of wild-type ERRα-1. As reported previously (3), the nuclear coactivator GRIP-1 augments the enhancer activity of ERRα-1 without the need of exogenous ligand. However, the F329A activity was not affected by the co-expression of GRIP-1 (Fig. 3). The latter result supports our prediction that Phe-329 is situated in the ligand-binding site. It is known that coactivators such as GRIP-1 interact with nuclear receptors at the ligand-binding domain when the receptor is in an agonist binding conformation. It was not expected that toxaphene, an antagonist for ERRα-1 (3), could act as an agonist for F329A (Fig. 4). Toxaphene increased the F329A activity in a dose-dependent manner, whereas the same compound decreased the wild-type activity. Therefore, the ERRα-1 mutant F329A becomes ERα-like in that it recognizes toxaphene as a weak agonist. However, GRIP-1 was found not to enhance F329A activity in the presence of toxaphene, indicating that GRIP-1 could not recognize toxaphene-bound F329A (results not shown).

To better understand the importance of Phe-329 in ERRα-1, we made and characterized the ERα mutant A350F. Our mammalian cell transfection experiments (in both HeLa and SK-BR-3 cells) have revealed that the ERα mutant A350F is transcriptionally active without the addition of E2 (Figs. 5 and 6).
In fact, E2 was not able to further increase the transactivation activity, indicating that E2 cannot bind to this ERRα mutant. Our experiments have also found that A350F can recruit GRIP-1 in the absence of ligands (Figs. 5 and 6). These results indicate that the ERRα mutant A350F becomes ERRα-1-like in that it is transcriptionally active in the absence of exogenous hormone.

Our yeast two-hybrid assay has found that the ERRα mutant A350F, but not wild-type ERRα, has the ability to transactivate a heterologous promoter such as GAL4 promoter. It was found that a chimeric receptor consisting of the DNA-binding domain of GAL4 fused to the ligand-binding domain of ERRα with the A350F mutation was transcriptionally active on a GAL4 reporter (Fig. 7). Ala-350 in ERRα is within an autonomous activation domain AF-2α (amino acids 282–351) (14).

X-ray structural analyses of the ERRα ligand-binding domain (10, 11, 15) have revealed that helix 12 assumes different conformations in response to ligand binding. In the presence of E2 and other agonists, helix 12 binds to a hydrophobic groove, the floor of which consists of ligands and side chains contributed by helices 3, 5/6, and 11. Antagonists such as tamoxifen partially fill the helix 12 groove, thus preventing helix 12 from assuming a conformation that recruits coactivator proteins (10). Results obtained from the analysis of the two mutants described in this paper allow us to conclude that, although we cannot exclude the possibility of the existence of a physiological ligand for ERRα-1, this orphan receptor is transcriptionally active in the absence of ligands. A molecular model of wild-type ERRα-1 reveals that the side chain of Phe-329 might be able to mimic bound ligand because it partially fills the binding pocket. This allows helix 12 to assume an active conformation capable of recruiting coactivator protein GRIP-1. As a result, the receptor is constitutively active (always turned on). This hypothesis has been evaluated by introducing a phenylalanine residue at the analogous position in ERRα (A350F). As shown in Fig. 8A, the phenyl side chain fills nearly half the estrogen binding cavity, thus completing the floor of the groove in which helix 12 normally binds when the agonist is present. As predicted, the A350F mutant is constitutively active.

Because the putative ligand cavity of wild-type ERRα-1 appears to be filled with side chains (most notably Phe-329), we predict that toxaphene binds in the hydrophobic groove normally occupied by helix 12 when it assumes the active conformation (Fig. 8B). Toxaphene therefore acts as an antagonist of wild-type ERRα-1, displacing helix 12 and preventing the recruitment of coactivator. However, when space is created in the putative ligand cavity via the mutation of Phe-329 to Ala, our model suggests that toxaphene can fill the cavity (Fig. 8C) and thus serve as an agonist, which it does when tested experimentally. However, the cavity is still not large enough to accommodate E2 or tamoxifen, which confirms our observation that these compounds are not ligands for wild-type ERRα-1 or the F329A mutant (data not shown). In addition, helix 12 does not assume a correct conformation for recruiting coactivator protein GRIP-1.

For classical receptors, ligand binding is essential for the transcription activity. Our results provide a structural basis as to why some orphan receptors such as ERRα-1 are transcriptionally active in the absence of exogenous hormone. In ERRα-1, the phenyl side chain of Phe-329 is situated in the ligand binding pocket that places the AF-2 domain in an active conformation. In addition, site-directed mutagenesis in the pro...
posed ligand binding pocket of ERRα-1 modifies the binding properties of toxaphene, strengthening the conclusion that this pesticide is a ligand of this receptor. Toxaphene is among the 12 persistent organic pollutants identified by the United Nations Environment Program as requiring urgent attention. Its antagonistic effect on ERRα-1 should not be overlooked.

In addition to Phe-329 in ERRα-1 and Ala-350 in ERα, there are two other nonconserved differences between the ligand-binding site of ERRα-1 and that of ERα. They are Gly-403 in ERRα-1, Phe-425 in ERα, Val-493 in ERRα-1, and Gly-521 in ERα (Fig. 1). The ERRα-1 mutant G403F has been generated and was found to have similar properties to the wild-type ERRα-1, indicating that this position is not as important as Phe-329. As expected, the function of the double mutant F329A/G403F is similar to F329A (results not shown). E2 was found not to be a ligand for the ERRα-1 mutant G403F or F329A/G403F. The ERRα-1 mutant V493G was also generated recently and found to be constitutively active. Neither E2 nor toxaphene acts as a ligand of this mutant. Although it is not unexpected that E2 is not a ligand of V493G, the results suggest that Val-493 is important for toxaphene binding. Additional mutagenesis experiments are being carried out to further characterize the role of Val-493 in toxaphene binding.

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