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Full-length estrogen receptor α and its ligand-binding domain adopt different conformations upon binding ligand

Ashok R. Bapat [a,*], Donald E. Frail [b]

[a] *Women's Health Research Institute, Wyeth Research, 500 Arcola Road, Collegeville, PA 19426, USA*
[b] *CNS Discovery Research, Pharmacia Corporation, Kalamazoo, MI 49007, USA*

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Abstract

The binding of ligand to a nuclear receptor causes conformational changes that can result in coactivator or corepressor recruitment and subsequent regulation of transcription. Several peptides have previously been identified that bind to the liganded estrogen receptor (ER). One interacting peptide, pepαII, was used in the present studies to assess the ability of ligands to induce spatial changes within both the full-length human estrogen receptor α (ER-α) and a truncated receptor containing the ligand-binding domain (LBD). pepαII interacted weakly with the full-length estrogen receptor α in the presence of both agonists and antagonists. In contrast, the interaction of pepαII with the truncated receptor containing the ligand-binding domain was strongly induced by antagonists and only weakly induced by agonists. Thus, the same ligand can induce different spatial configurations of the full-length and ligand-binding domain of estrogen receptor α as measured by pepαII affinity. Crystal structures of nuclear hormone receptors solved to date have used ligand-binding domains and therefore may not accurately predict surface interaction domains present in the liganded full-length receptor. Furthermore, the ability of a ligand to induce a strong interaction of pepαII with the estrogen receptor α ligand-binding domain predicts that the ligand will have greater antagonist activity on the full-length receptor.

Keywords: Estrogen receptor α; Interaction with ligand; Conformational changes

1. Introduction

Estrogens exert numerous biological effects in a wide variety of tissues by binding to and activating estrogen receptors α and β (ER-α and β), two highly related members of a large superfamily of ligand activated transcription factors [1]. Upon binding ligand, the estrogen receptor undergoes conformational changes that allow the binding of the receptor to specific DNA response elements and reveal sites of interaction for transcriptional coactivators or corepressors, thereby altering the expression of specific genes. Estrogens can have different biological activities in different tissues. For example, tamoxifen is an antagonist in breast tissue, but an agonist in bone and uterine tissue [2–4]. A number of compounds with similar tissue selective activity have since been described and are referred to as "selective estrogen receptor modulators" (SERMs) [5]. The mechanisms by which a ligand can mimic the activity of 17β-estradiol in one tissue and antagonize this activity in a different tissue are under intense investigation. While the discovery of a second estrogen receptor (ER-β) several years ago presented a potential explanation [6], it has become apparent that receptor subtypes cannot fully explain the tissue selective actions of ER ligands. A second hypothesis to explain tissue selectivity is that ligands selectively recruit a subset of coactivators or corepressors to the receptor to enhance or inhibit transcription of specific genes, respectively. In recent years, a number of proteins have been reported to interact with the ER in the presence of ligand and modulate its transcriptional activity [7]. It is hypothesized that a differential recruitment of coactivators and/or corepressors (which themselves may be selectively expressed in different tissues) may occur in response to the receptor conformation induced by a given ligand and thereby give rise to the observed tissue selective activities of SERMs [8].

Numerous biochemical studies have provided a basic understanding of the relationship between structure and function of the ER. The receptor structure includes an amino terminal domain containing an activation function (AF-1), a central DNA binding domain (DBD), sequences downstream of the DNA binding domain contain the ligand-binding domain (LBD) and a second, stronger activation function (AF-2) [9].

* Corresponding author. Tel.: +1-484-865-2454; fax: +1-484-865-9367.
E-mail address: bapata@wyeth.com (A.R. Bapat).

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More recently, crystal structures of liganded ER-α [10–12] and ER-β [13] have been reported, thus defining key elements of receptor structure. For technical reasons, all crystals obtained have been of truncated ER proteins containing only sequences downstream of the DBD, including the LBD. Nevertheless, these studies have defined the key amino acids involved in the binding of ligand and have identified conformational states induced by different ligands. For example, the conformations of ER-α induced by the agonist 17β-estradiol and the SERM raloxifene are clearly different [10]. Moreover, the crystal structure for the complex formed between diethylstilbestrol, ER-α and a coactivator fragment [12] has confirmed one site of receptor–coactivator interaction that was predicted from earlier biochemical and mutation studies [14] and has provided a structural rationale for the activity of agonists and antagonists.

Crystal structures, although offering the highest resolution view of structure, provide a static view of the receptor. Ligand induced conformational changes have also been indirectly investigated using peptides identified through phage display [15,16]. Using the full-length ER-α, peptides were identified that interact with the receptor in the presence of various ligands. For example, one peptide, referred to as α/βIII, interacted with the full-length receptor only in the presence of tamoxifen, thus identifying a unique receptor conformation induced by this compound. A second peptide, referred to as αII (pepαII) interacted weakly with full-length receptor in the presence of both agonists and antagonists, thus apparently identifying a structural motif induced by all ligands tested. Herein, pepαII was used to assess the ability of compounds to induce conformational changes in both full-length ER-α and a truncated receptor containing the LBD analogous to that used to prepare crystals for structural analysis. Because the full-length receptor and the LBD bind 17β-estradiol with equal affinity, it has been assumed that the full-length receptor and the LBD adopt similar conformations upon binding ligand. However, the present results indicate that full-length ER-α and ER-α–LBD adopt different conformations in the presence of the same ligand. Since the conformation of the SERM-receptor complex dictates the biological activity of these SERMs, interpretation of data obtained using LBD and then extending those findings to the full-length receptor should be interpreted with caution.

2. Materials and methods

All compounds were obtained from the Wyeth compound library and solubilized in DMSO. All chemicals were reagent grade and were purchased from Sigma–Aldrich, St. Louis, MO.

Mammalian two-hybrid constructs were obtained by subcloning PCR-synthesized ER-α sequences, or the pepαII sequence, into the *Eco*RI and *Mlu*I sites of pVP16 (Clontech, Palo Alto, CA) and the *Eco*RI and *Bam*HI sites of pM (Clontech), respectively. Oligonucleotides containing the necessary restriction enzyme sites were used to obtain full-length ER-α and a truncated region containing the ligand-binding domain of ER-α (K303-V595) by PCR using a plasmid template containing the authentic human ER-α coding sequence. The PCR products and digested vector were gel purified, ligated, and the appropriate clones were confirmed by sequencing. To obtain the pepαII construct, overlapping oligonucleotides were prepared that encoded the necessary restriction sites and the sequence of pepαII (SSLTSRDFGSWYASR). The oligonucleotides were annealed and ligated to digested pM vector containing the Gal4 DNA binding domain. The sequence of the resulting plasmid used was confirmed. A luciferase reporter plasmid, pGL3-220 containing five copies of the Gal4 UAS (Promega Inc., Madison, WI), and a β-galactosidase (β-Gal) reporter plasmid, SV-40/β-Gal, were used to assess transfection efficiency.

2.1. CELL culture and transfection

COS-7 (ATCC #CRL1651) cells and HepG2 (ATCC #HB8065) cells were maintained overnight in phenol red-free DMEM/deficient growth medium (Bio-Whittaker 12917-F) containing 1× each of MEM non-essential amino acids, Pen/Strep, Glutamax-1 supplemented with heat inactivated, charcoal stripped, 10% fetal bovine serum.

Cell lines were transfected using Lipofectamine-2000 transfection reagent according to the supplied protocol (GIBCO-BRL Life Technologies, Rockville, MD). Briefly, lipofectamine 2000 was diluted in Opti-MEM I medium (1:48) and allowed to incubate for 5–15 min. Simultaneously, the DNA mixture was prepared. Typically, a mixture for nine wells of a 96-well plate contained 0.5 μg of ER-α–LBD/VP16 plasmid, 0.5 μg of pepαII/pM plasmid, 0.025 μg of CMV/β-Gal plasmid, and 0.25 μg of the pGL3-220 luciferase reporter plasmid in 240 μl of Opti-MEM I. The DNA mixture was added to an equal volume of transfection reagent and incubated for 20 min at room temperature. Cells were trypsinized and then resuspended in media to a concentration of 10^6 cells/ml. The incubated DNA mixture was added to the appropriate volume of cells (50,000 cells per well), brought to a final volume of 150 μl per well with media, plated into wells of a 96 well plate, and incubated overnight (18 h) at 37 °C.

The following day, the medium was removed and replaced with fresh media containing test compounds in DMSO. The final concentration of DMSO in the wells was ≤0.1%. The cells were incubated overnight (18 h), washed with phosphate buffered saline, and lysed by adding 100 μl of 1× reporter lysis buffer (Promega Inc., Madison, WI). Following one freeze thaw, 10 μl of lysate was used to measure luciferase activity (Promega Inc., Madison, WI) and 5 μl was used to measure β-galactosidase activity according to the supplied protocol (Tropix, Bedford, MA). Each compound was tested in quadruplicate. For dose-response curve fitting, a four-parameter logistic model on the transformed,

weighted data was fit and the IC_{50} was defined as the concentration of compound decreasing maximum activity by 50%.

Control transfections were performed to assess the activity of each individual two-hybrid plasmid. Background activities were observed with pepαII/pM and the pVP16 plasmid, as well as with either ER-α full-length or ER-α–LBD/pVP16 and the pM plasmid in the presence of 17β-estradiol (data not shown).

3. Results

A mammalian two-hybrid system was used to assess the interaction between ER-α and pepαII (SSLTSRDFGSWYASR). Constructs containing full-length ER-α or ER-α–LBD fused to the viral transactivator VP16 and pepαII fused to the Gal4 DNA binding domain were used. The interaction of pepαII with ER-α resulted in the recruitment of the VP16 transactivator to the Gal4 DNA response element and a subsequent increase in reporter gene activity.

In the presence of 1 μM of ligand, full-length ER-α interacted with pepαII similarly in both HepG2 and COS cells (Fig. 1A and B). The extent of the interaction was moderate as shown by a 5–15-fold increase in reporter gene activity. Both agonists and antagonists induced this interaction and there was not an obvious differentiation among the agonists and antagonists tested, consistent with previous reports [15,16]. The spectrum of compounds tested included full agonists (17β-estradiol, 17α-ethinyl estradiol), full antagonists (EM 800, ICI-182780), and SERMs (4-OH-tamoxifen (4-OH-Tam), raloxifene). When a scrambled peptide (amino acid composition was the same as that of pepαII) fused to Gal4 DNA binding domain was used in the two-hybrid assay, there was no increase in the reporter gene activity. This suggested that the interaction between ER-α–LBD or FL and pepαII was pepαII sequence specific and ligand dependent.

Liganded ER-α–LBD also interacted with pepαII in both HepG2 and COS cells (Fig. 2). However, in contrast to the results obtained with full-length ER-α, the degree of the interaction was very large in some cases, as shown by an increase in reporter gene activity of >1000-fold. In addition, while both agonists and antagonists induced the interaction, the antagonists were clearly more active. Of note, ICI-182780 and EM 800 [17], two ligands often considered to be “pure” antagonists, consistently gave the most robust activity. In contrast, the agonists 17β-estradiol and 17α-ethinyl estradiol consistently exhibited relatively low activity. Raloxifene, a SERM, consistently had intermediate levels of activity.

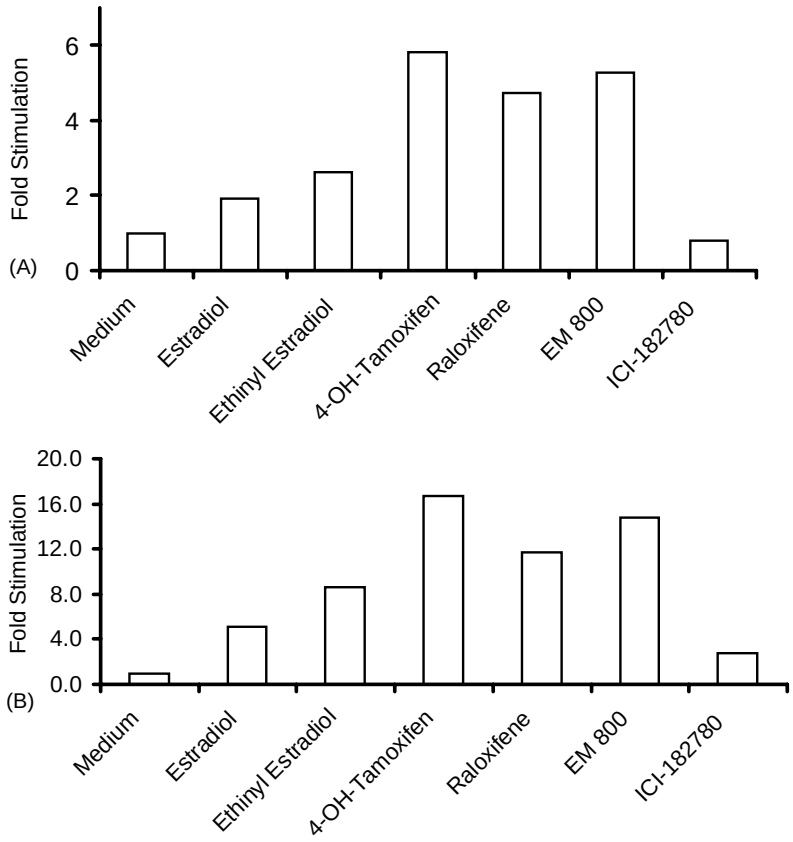

Fig. 1. Full-length ER-α interaction with pepαII as measured by a mammalian two-hybrid assay in HepG2 (A) and COS (B) cells. The concentration of each ligand used was 1 μM. The data are plotted as the fold induction compared to activity in the absence of ligand (medium alone). Each experiment was performed in triplicate and the data shown represents the mean of two independent experiments.

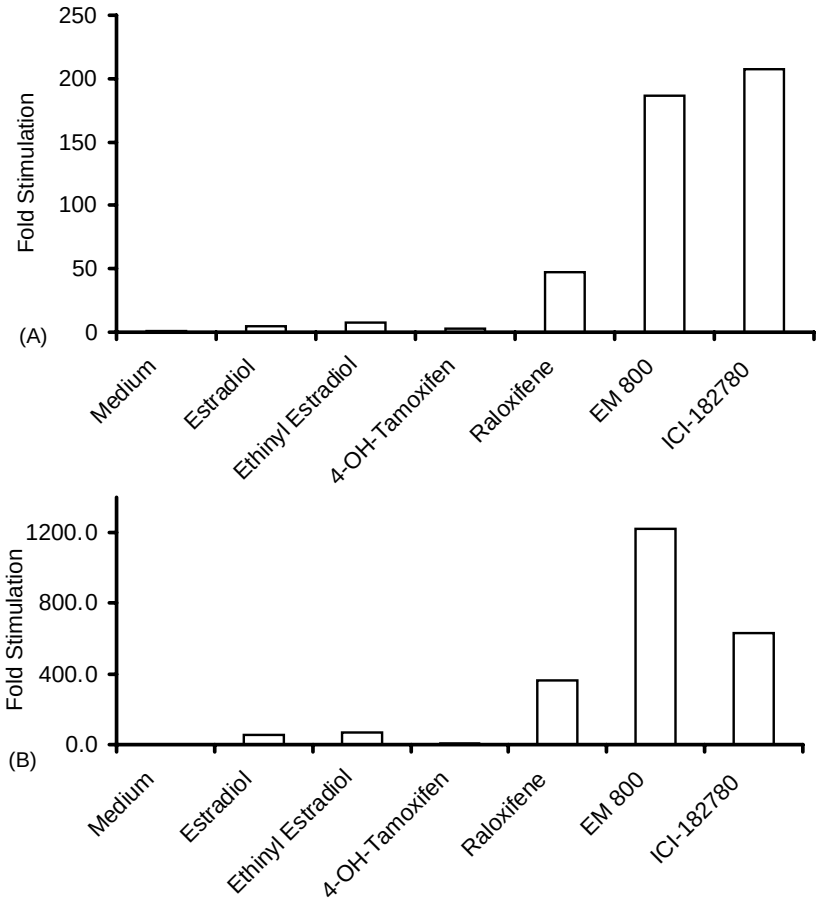

Fig. 2. ER-α–LBD interaction with pepαII as measured by a mammalian two-hybrid assay in HepG2 (A) and COS (B) cells. The concentration of each ligand used was 1 μM. The data are plotted as the fold induction compared to activity in the absence of ligand (medium alone). Each experiment was performed in triplicate and the data shown represents the mean of two independent experiments.

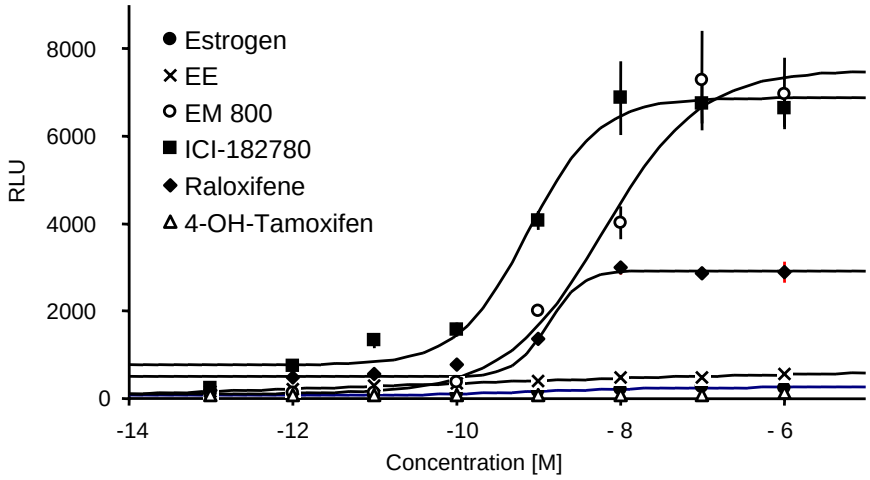

Fig. 3. A mammalian two-hybrid assay in COS cells measuring the ability of various ligands to induce the interaction of ER-α–LBD with pepαII. Concentration (M) response curves for a number of agonists (estrogen (●), ethinyl estradiol (×)) and antagonists (ICI-182780 (■), EM 800 (○)), including SERMs (4-OH-tamoxifen (△), raloxifene (◆)) are shown. Every experiment was performed in triplicates and repeated once. The data are expressed as mean + S.D. and plotted as relative light units (RLU) of normalized reporter gene activity.

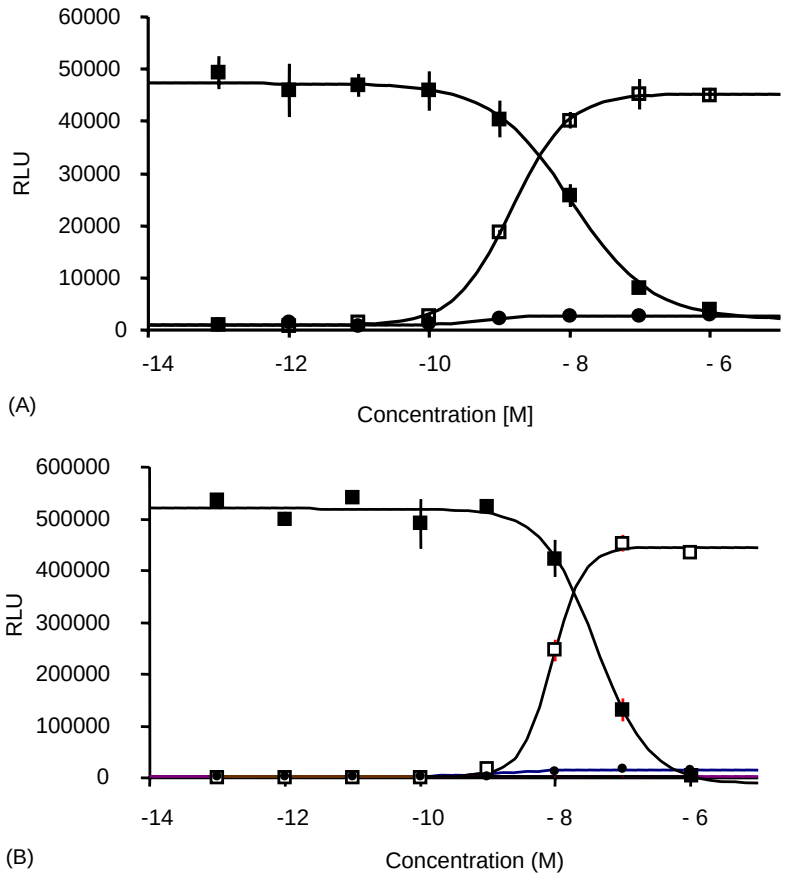

Fig. 4. A mammalian two-hybrid assay in COS cells measuring the ability of a ligand to inhibit the interaction of ER-α–LBD with pepαII induced by ICI-182780. Various concentrations of 17β-estradiol (A) or 4-OH-tamoxifen (B) were used to compete the interaction induced by 100 nM (■) of ICI-182780. The concentration-responses for ICI-182780 (□) and 17β-estradiol or 4-OH-Tam (●) were determined in the same assay. The data are plotted as relative light units (RLU) of normalized reporter gene activity. Every experiment was performed in triplicate and repeated once. The data are expressed as mean + S.D. and plotted as RLU of normalized reporter gene activity.

Surprisingly, 4-OH-tamoxifen, also a SERM, gave little to no activity in the assay using the ER-α–LBD, while it was active using the full-length ER-α (Fig. 1).

The ability of various compounds to induce the interaction of ER-α–LBD over a range of concentrations was determined to be sure that the differences observed among compounds in their ability to induce the interaction of ER-α–LBD with pepαII was not an artifact of concentration (Fig. 3). In all cases, a concentration response relationship was observed and maximal activity was obtained at 0.1 μM.

Finally, the ability of ligands to reverse the interaction induced by ICI-182780 was determined. At two different concentrations of ICI-182780, 17β-estradiol inhibited the interaction of ER-α–LBD with pepαII in a concentration-dependent manner (Fig. 4A). When tested alone, 17β-estradiol caused a relatively low level of interaction, a level also obtained when high concentrations of 17β-estradiol were used to compete for ICI-182780. As previously shown (Fig. 2), tamoxifen induced the interaction of pepαII with ER-α–LBD very weakly or not at all. However, tamoxifen inhibited the interaction of ER-α–LBD with pepαII induced by ICI-182780 in a concentration-dependent manner (Fig. 4B).

4. Discussion

Nuclear receptor mediated transcription is now known to be modulated through the interaction of the receptor with various coactivators and corepressors [7,8]. The binding of ligand to the receptor induces conformational changes that mask or reveal sites of interactions for these modulatory proteins. For example, ER-α undergoes conformational changes upon the binding of an agonist that reveals a binding pocket for the p160 family of coactivators, including SRC-1, TIF-2 and SRC-3 [12]. However, evidence is mounting that ER-α (and other nuclear receptors) is not a molecular on/off switch governed by two conformations, one induced by agonists and the other induced by antagonists. Rather, the receptor appears to be a molecular rheostat, adopting a spectrum of conformations that result in the variety of observed tissue selective activities [18].

Phage peptide display has been used to identify peptides that recognize various conformations of full-length ER-α induced by different ligands [1,6,15]. A number of peptides have been reported to interact with ER-α in a ligand dependent manner. While many of these peptides have a LXXLL amino acid motif that is essential for the receptor interaction domain of the p160 family of coactivators [14], some interacting peptides do not contain this motif, suggesting that there may be additional sites for peptide (and perhaps coactivator) interactions on the receptor. In particular, one peptide, referred to as αII (pepαII), does not contain a LXXLL motif and was reported to interact with full-length ER-α in the presence of ligand [15,16]. The extent of the interaction was similar for all ligands tested, including both agonists and antagonists. In this study, the conformational states of ER-α induced by different ligands were further probed by assessing the ability of a particular ligand to induce the interaction of pepαII with ER-α. In addition to full-length ER-α, a truncated form of ER-α that contained the ligand-binding domain (ER-α–LBD) was also used because the crystal structures reported to date for ER-α used similar truncated constructs [10–13]. The conformational structures induced by various ligands determined by these crystal structures are generally assumed to apply to the full-length receptor. However, one cannot rule out a possibility that the conformation of the LBD that is induced by ligand binding is the same in full-length ER-α as well as truncated ER (containing only the LBD). In that case other domains of the receptor may influence the ability of the fusion proteins to bind to the liganded receptor.

Similar to previous reports, the interaction of pepαII with full-length ER-α was rather weak and was not remarkably different among the agonists or antagonist tested [15,16]. However, significant differences were observed among various ligands in their ability to induce the interaction of ER-α–LBD with pepαII. The interaction of the ER-α–LBD, but not the full-length receptor, was strongly induced by the antagonists ICI-182780 and EM 800, indicating that the ER-α–LBD adopts a conformation somewhat different from the full-length receptor. Furthermore, the extent of the ligand induced interaction of ER-α–LBD with pepαII covers a wide spectrum, from little to no interaction with tamoxifen, to weak interaction with 17β-estradiol or 17α-ethinyl estradiol, to stronger interaction with raloxifene, to very strong interaction with ICI-182780 or EM 800. The spectrum of activities observed is consistent with the hypothesis that the receptor acts as a molecular rheostat, adopting a spectrum of conformations upon binding different ligands [18]. Furthermore, the ability of a ligand to induce the interaction of ER-α–LBD with pepαII provides an assay system to assess the conformation induced by the ligand.

It is interesting to note that the ability of a compound to recruit pepαII to ER-α–LBD is generally consistent with the amount of known agonist/antagonist potential of the compound in-vivo. That is, those compounds that are generally agonists (“pure” agonists) have a low amount of activity, and those compounds that are generally antagonists in all tissues (“pure” antagonists), have a high amount of activity. Raloxifene, a SERM that has tissue dependent agonist or antagonist activity, consistently gave intermediate levels of activity. Tamoxifen, also a SERM, is clearly an exception. Tamoxifen induced little to no activity, indicating that it induces a receptor conformation quite different from other SERMs. Norris et. al. previously identified a peptide, α/βIII, that interacts with the full-length ER-α only in the presence of tamoxifen, also suggesting that tamoxifen induces a unique conformation [16]. Clearly, the ability of a compound to induce certain conformations of ER-α can be measured by its ability to induce an interaction with pepαII. Furthermore, the ability of a compound to recruit ER-α–LBD to pepαII is consistent with the known stimulatory activity of a compound on the endometrium. Compared to17-β estradiol, 4-OH-tamoxifen (and tamoxifen) gave the same increase in epithelial cell height, while raloxifene gave a 50% increase and ICI-182780 gave no increase [19].

The recent resolution of crystal structures of ER-α have identified the key amino acids involved in the ligand-binding site and have revealed the basic structure changes that lead to agonist or antagonist activity [10–13]. These studies have relied on the use of truncated proteins quite similar to the ER-α–LBD used here. However, our results provide evidence that the conformations induced by various ligands are different between the ER-α–LBD and the full-length receptor. Although the truncated receptor provide valuable information regarding its interaction with ligands, caution is warranted when extrapolating these results obtained from the binding studies using the ER-α–LBD to the full-length receptor. Furthermore, the crystal structures provide a higher resolution single frame snapshot of the receptor, and additional studies, including the peptide interaction studies, are needed to provide a dynamic view of receptor shape in real time.

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