Different Residues of the Human Estrogen Receptor Are Involved in the Recognition of Structurally Diverse Estrogens and Antiestrogens

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We have previously examined, by alanine scanning mutagenesis, amino acids 515–535 of the estrogen receptor (ER) ligand binding domain to determine which of these residues are important in estradiol binding. Mutation at four sites that potentially lie along one face of an α-helix, Gly521, His524, Leu525, and Met528, all significantly impaired estradiol binding by the ER (Ekena, K., Weis, K. E., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (1996) J. Biol. Chem. 271, 20053–20059). In this report, we compare the pattern of residues that are important in the recognition of several structurally diverse estrogen agonists and antagonists (the synthetic nonsteroidal agonist hexestrol, an agonist derived from the mold metabolite zearalenone, P1496, and the partial agonist-antagonist trans-hydroxytamoxifen) with those that are predicted to contact estradiol in the receptor-ligand complex. Although there are some similarities in the pattern of residue recognition among all four ligands, each ligand showed distinct differences as well. Interestingly, alanine substitution at only one residue, the leucine at position 525, was found to inhibit binding of all the ligands tested. Another residue, His524, was found to be important in the recognition of three different agonists but not trans-hydroxytamoxifen (the only ligand lacking a second hydroxyl group). The recognition of estradiol and another agonist, P1496, was impaired by the G521A mutation, whereas ligand-induced activity by the two compounds that lack B- and C-rings, hexestrol and trans-hydroxytamoxifen, was unaffected.

Our findings demonstrate that these ligands fit into the ER ligand binding pocket differently and that each contacts a distinct set of amino acids. The smaller ligands (estradiol and hexestrol) have a narrower footprint of interacting residues than the larger ligands (P1496 and trans-hydroxytamoxifen). This pattern of interaction is most consistent with the amino acids within this region being in contact with the portion of these ligands that corresponds to the D-ring end of estradiol. The interplay between the shape of an ER ligand and the residues that support its binding to ER may potentially underlie the selective actions of different ER ligands in various cell and promoter contexts.

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§ The costs of publication of this article were fully used in the treatment of ER-positive breast cancers (3, 12, 13). These antagonists compete with estradiol for binding to the receptor but fail to elicit transcriptional activity. Unfortunately, tamoxifen treatment has several drawbacks. First, tamoxifen is a mixed agonist and antagonist and has agonistic effects in certain cell and promoter contexts. Second, breast cancer cells often become resistant to this treatment (14). Finally, despite the need to inhibit ER activity in breast cancer cells, the retention of some ER activity is important in a number of other biological processes, including bone maintenance, the cardiovascular system, and liver metabolism (2). Thus, it becomes important to understand how the ER recognizes and interprets ligands of different structure, that is, which amino acids are involved in their binding, and thereby how these ligands might result in different activities in various cell types. Such information could then be used to design new estrogen agonists and antagonists with target cell- and response-selective biological effects.

We and others have identified regions of the ER involved in recognition of ligand (15–18). We have found amino acids near Cys530 to be important in estradiol binding, whereas residues C-terminal to position 535 have been shown to contain AF-2 activity (19–21), and residues 507–514 may contain dimerization activity (20, 22). Recently, we used alanine scanning mutagenesis to identify those amino acids in the 515–535 region of the ER involved in recognition of estradiol. This work was supported by National Institutes of Health Grants 2R37CA68653 (to K. E.) and 2R37DK15556 (to J. A. K.) and in part by National Institutes of Health Postdoctoral Fellowship 1 F32CA68653 (to K. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

The abbreviations used are: ER, estrogen receptor; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; ERE, estrogen response element; ICI, ICI 164,384; RA, retinoic acid; RAR, retinoic acid receptor; T3, thyroid hormone; TOT, trans-hydroxytamoxifen; WT, wild type.
the ER ligand binding domain important in estradiol binding (23). Mutation of the amino acids Gly521, His524, Leu525, and Met528 to alanine affected the ability of the ER to bind estradiol and had a parallel effect on estrogen-dependent transcription without affecting the overall activity of the receptor. These results, taken together with structural predictions based on crystal structures of other ligand-bound nuclear receptors, indicated that these four amino acids of the ER may lie along one face of an α-helix and are presumed to be in contact with the bound estradiol.

In this report, we extend those studies to include four other estrogen agonists and antagonists with varied structures: the synthetic nonsteroidal agonist meso-hexestrol; P1496, an agonist derived from the mold metabolite zearalenone; the partial agonist-antagonist trans-hydroxytamoxifen; and the pure antagonist ICI. Interestingly, we observe that different amino acids in the 515–535 region of the ER are important in the recognition of these different ligands. Only one amino acid (Leu525) was found to be important in binding all of the ligands. By comparing the amino acids important in binding each of the ligands and comparing the ER amino acid sequence with other nuclear receptors and published crystal structures, we suggest which portion of these ligands is in contact with this region of the ER ligand binding pocket.

MATERIALS AND METHODS

**Plasmids, Ligands, and General Reagents**—The plasmids (ERE)2-pS2-CAT (24), pCMV5hER (19), pCMVβ (Clontech, Palo Alto, CA), and pTZ19R (25) have been described. Plasmid DNA used for transfection was purified either by CsCl gradient centrifugation (26) or by Qiagen plasmid preparation kit according to the manufacturer’s instructions (Qiagen, Chatsworth, CA). Ligands were from the following sources: meso-hexestrol and 17β-estradiol (Sigma); P1496 (kindly provided by IMC Corp., Terre Haute, IN); and trans-hydroxytamoxifen and ICI (kindly provided by Dr. Alan Wakeling and Zeneca Pharmaceuticals, Macclesfield, UL). Cell culture medium, calf serum, and other reagents (kindly provided by Dr. Alan Wakeling and Zeneca Pharmaceuticals, Macclesfield, UL). Cell culture medium, calf serum, and other reagents were purchased from Life Technologies, Inc. and Sigma.

**Cell Culture and Transfections**—All transfections were performed using the ER-negative human breast cancer cell line MDA-MB-231. Cells were maintained as described previously (10) and were transfected using the calcium phosphate method (27). Cells were incubated in 5% CO2 for 40–48 h prior to transfection with 0.1 μg of ER expression plasmid, 2.0 or 5.0 μg of (ERE)2-pS2-CAT reporter plasmid, 0.8 μg of pCMVB internal control β-galactosidase plasmid, and pTZ19R to 15 μg of total DNA/100-mm plate. After incubation of cells with DNA for 4 h, cells were glycerol shocked for 2.5 min with 20% glycerol in growth medium and washed with Hanks’ balanced salt solution for 2.5 min. Cells were then treated with ligand in growth medium, harvested after 24 h, and lysed by cycles of freezing on dry ice and thawing at 37 °C. ER activity was determined by CAT assay of whole cell lysates and was normalized to β-galactosidase activity described previously (28).

RESULTS

**Ligand-induced Responsiveness of Alanine-substituted Human Estrogen Receptor Mutants to Estrogen Agonists and Antagonists**—The mutant receptors used in this study, which consist of individual alanine substitutions from amino acid 515 to amino acid 535 of the ER ligand binding domain, were described by us (23) in a study in which their ability to bind estradiol and activate transcription in response to estradiol was analyzed. The responsiveness of these mutant receptors to two different estrogen agonists, hexestrol and P1496, the partial agonist/antagonist, trans-hydroxytamoxifen (TOT), and a pure antagonist, ICI, was investigated in this study. The structures of these ligands are shown in Fig. 1. The ability of these ligands, all of which have high affinity for the ER (29–31), to induce or antagonize transcriptional activity of the mutant receptors was monitored in the ER-negative breast cancer cell line MDA-MB-231 using an estrogen-responsive reporter gene construct, (ERE)2-pS2-CAT, in transient cotransfection experiments. As expected for wild type ER, both hexestrol and P1496 fully induced transactivation to near estradiol-induced levels (Fig. 2A). TOT induced activity to only 10–15% that of estradiol, and it antagonized estradiol-induced activity down to its own level of agonist activity (10–15% Fig. 2C). ICI, as expected, behaved as a pure antagonist (Fig. 2B).

To assess the importance of the individual amino acids from positions 515–535 in the recognition of these receptor ligands, the mutant ERs were initially screened at a single concentration of each ligand that resulted in near maximal stimulation of wild type (WT) ER. Mutants whose activity differed significantly from wild type at this concentration were then subsequently tested over a range of ligand concentrations to determine whether the mutant receptors were dose-shifted in their response to ligand, suggesting a decreased affinity for ligand, or if they were defective in the ability to achieve maximal transcriptional activity. For all of these receptors, activity was low in the absence of ligand, as reported previously (23).

**Hexestrol**—The structure of the synthetic nonsteroidal estrogen hexestrol (Fig. 1) differs from estradiol in that it is symmetrical and lacks a formal B- or C-ring. The mutant ERs were initially screened at 1 × 10−10 M hexestrol, which gave 75% of maximal activity with WT ER. As shown in Fig. 3A, alanine
substitutions at only two positions, His524 and Leu525, resulted in large reductions in hexestrol-induced transactivation (5% of WT activity). The R515A, M528A, and P535A receptors exhibited modest reductions in activity (60–80% of WT). Dose response curves for the WT, H524A, L525A, and P535A receptors using 1 × 10⁻¹² to 1 × 10⁻⁶ M hexestrol, are shown in Fig. 3B. The most impaired mutant, H524A, was dose-shifted 1000-fold, whereas L525A and P535A were shifted 10³ and 10², respectively. The R515A and M528A receptors were also dose-shifted slightly, requiring slightly higher levels of hexestrol to reach wild type maximal activity (data not shown). Though His524 and Leu525 appear to be important in both estradiol and hexestrol binding by ER (Table I), alanine substitution at position 524 had a much greater effect on hexestrol-induced activation. Interestingly, the G521A mutation, which was found to significantly affect estradiol binding (Table I), had very little effect on hexestrol-induced transactivation (Fig. 3C).

P1496—P1496, a derivative of the estrogenic mold metabolite zearalenone, has a 14-membered ring resorcylic acid lactone structure that is significantly larger than either estradiol or hexestrol (Fig. 1). Nevertheless, it acts as a potent inducer of ER activity, stimulating ER transactivation to nearly the same level as estradiol (Fig. 2). Like hexestrol, 1 × 10⁻¹⁰ M P1496 gave near maximal (80%) stimulation of ER and was therefore used to screen the 21 alanine-substituted ER mutants. Although mutation to alanine at only two positions markedly impaired hexestrol-induced activity (Fig. 3A), half of the ER mutants were substantially impaired for P1496-induced activity (Fig. 4A). Using higher concentrations of ligand demonstrated that all of these mutant receptors could reach wild type maximal transactivation at higher concentrations of ligand (Fig. 4B and C, and data not shown). Like estradiol induction, the positions where mutation to alanine most greatly affected P1496-induced transactivation were Glu521, His524, Leu525, and Met528. However, in addition, mutation at residues 515A, 522A, 526, and 531–535 also had a significant effect on P1496-induced activity. Fig. 4 (B and C) shows the P1496 dose response curves for the R515A, G521A, M522A, H524A, L525A, and M528A receptors. Of particular note is the observation that the M528A mutation affects receptor response to P1496 more than this mutation affects response to any of the other tested li-
The presence of higher concentrations of ligand (Fig. 5) allowed the truncated receptors to reach wild-type levels of activity in the context of partial agonistic activity in certain cell types, including MDA-MB-231 cells (Fig. 2). To investigate the ability of the mutant ERs to interpret TOT as an agonist, transient transfections were performed with hexestrol and P1496, except that more reporter plasmid, 5 µg rather than 2 µg, was used in order to enhance the agonistic effects. The results of screening the mutant receptors with 1 × 10⁻³ M TOT are shown in Fig. 5A and show that like P1496, half of the mutant receptors were impaired for activity. However, unlike the P1496-induced response, TOT dose response analysis revealed two different classes of mutants. As observed before, many of the mutants that were impaired for activity at the initial concentration of TOT were able to reach wild type levels of activity in the presence of higher concentrations of ligand (Fig. 5B and data not shown). However, another class of mutants, which consisted of K520A, M522A, N532A, and P535A (and indicated with light shading in Fig. 5A), all exhibited reduced maximal activity and did not appear to be dose-shifted (Fig. 5C and data not shown). Rather, the decreases in activity observed at 1 × 10⁻³ M TOT correlated with their reduced maximal activities. Western blot analysis of these receptors demonstrated that they were present at levels at least as great as that of the wild type protein (data not shown), indicating that their reduced activity was not attributable to reduced levels of these receptors.

**Antagonists**—The antagonistic affects of two compounds, TOT and ICI, were examined to determine whether any of the alanine-substituted receptors failed to recognize these ER ligands as antagonists. For these experiments, cells were treated with both estradiol and a 10-fold excess of either TOT or ICI. For most of the mutant receptors, 1 × 10⁻⁹ M estradiol with 1 × 10⁻⁸ M antagonist was used. However, for G521A, H524A, and L525A, where 1 × 10⁻⁹ M estradiol does not induce high levels of transactivation (23), 1 × 10⁻⁸ M (for G521A and H524A) and 1 × 10⁻⁷ M (for L525A) estradiol were used with a corresponding 10-fold higher concentration of the antagonist. In all cases, both TOT and ICI were effective antagonists, suppressing estradiol-induced transactivation by greater than 85% (data not shown).

**DISCUSSION**

We have probed the interaction that various ligands have with a portion of the ligand binding domain of the human estrogen receptor (ER) by alanine scanning mutagenesis, studying in addition to estradiol the behavior of three high affinity ligands: two nonsteroidal agonists, the simple, symmetrical synthetic ligand meso-hexestrol, and P1496, a derivative of the fungal produced estrogen zearalenone, as well as the partial antagonist, trans-hydroxytamoxifen, the potent metabolite of tamoxifen. As in our earlier study with estradiol, we found that the substitution of certain residues in this ER region to alanine caused a significant to marked reduction in the potency of these ligands in inducing transcriptional activity. In addition, the principal finding from this study is that each of the ligands displayed a distinct footprint of interacting resi-

### Table I

**Comparison of amino acids important in the recognition of estrogen receptor ligands**

The number of circles represents the level of impaired ligand recognition by the mutant receptors; more circles indicate greater impairment. Open circles represent mutant receptors that have decreased maximal activity.

| Receptor | Ligand | Estradiol | Hexestrol | P1496 | TOT |
|----------|--------|-----------|-----------|-------|-----|
| R515A    |        |           |           |       |     |
| H516A    |        |           |           |       |     |
| M517A    |        |           |           |       |     |
| S518A    |        |           |           |       |     |
| N519A    |        |           |           |       |     |
| K520A    |        |           |           |       |     |
| G521A    |        |           |           |       |     |
| M522A    |        |           |           |       |     |
| E523A    |        |           |           |       |     |
| H524A    |        |           |           |       |     |
| L525A    |        |           |           |       |     |
| L526A    |        |           |           |       |     |
| S527A    |        |           |           |       |     |
| M528A    |        |           |           |       |     |
| K529A    |        |           |           |       |     |
| C530A    |        |           |           |       |     |
| K531A    |        |           |           |       |     |
| N532A    |        |           |           |       |     |
| V533A    |        |           |           |       |     |
| V534A    |        |           |           |       |     |
| P535A    |        |           |           |       |     |

*C* Open circles represent mutant receptors that have decreased maximal activity.

**Fig. 4.** P1496-induced activity of the wild type and mutant estrogen receptors. MDA-MB-231 cells were transfected as described in Fig. 3 except that the compound P1496 was used instead of hexestrol. *A*, bars represent the level of P1496-induced activity for all the mutant receptors at 1 × 10⁻¹⁰ M ligand. Wild type ER activity at this concentration is set to 100%. *B* and *C*, dose response curves using 1 × 10⁻¹² to 1 × 10⁻⁶ M P1496, for the wild type, R515A, M522A, L525A, G521A, H524A, and M528A receptors. The R515A, M522A, and L525A receptors are dose-shifted 10–20-fold relative to wild type ER in their response to P1496. The M528, G521A, and H524A receptors are shifted 50-, 400-, and 1000-fold, respectively. All values represent the means and standard deviations of two or more experiments. For some values, error bars are too small to be seen.
Estrogen Receptor Ligand Interaction Sites

In this study, we find that the pattern of residues in the 515–535 region of ER that affect ligand-induced transcription differs depending on the structure of the activating ligand. A comparison of the structures of these ligands (Fig. 1) together with their interacting residues (Table I) allows one to formulate a model for the basic orientation of these ligands within the binding cavity of the ER (Fig. 6). By analogy with the TR-T3 and RAR-RA structures, this model shows the 515–535 region of ER as a helical region (corresponding to a portion of helix-10 and all of helix-11) and the beginning of the loop region between helix-11 and -12. The residues that affect the transcriptional potency of each of the four ligands are indicated schematically with circles. The helix and loop of ER and the structural scale to illustrate the differential interaction that these four ligands have with the ER.

The four activating ligands, estradiol, hexestrol, P1496, and TOT, all have one common structural feature, aphenol, yet they show different patterns of residues whose substitution with alanine affects their transcriptional potency. These differences suggest that the ligands are not making contact with the 515–535 portion of the ER through their structurally common A-ring like feature. The rather narrow footprint of residues that affect hexestrol recognition (principally two residues, His524 and Leu525, on one turn of the helix) suggests that...
contact is being made with a portion of the hexestrol structure that is narrower than the corresponding region in estradiol (whose potency is affected principally by three residues, Gly\textsuperscript{521}, His\textsuperscript{524}, and Leu\textsuperscript{525}, on two helical turns). In contrast, the much wider footprint of residues that affect P1496 binding (four or more residues on at least three turns) suggests that their interaction is with a region of the ligand that is larger than the corresponding region in estradiol.

Thus, if the phenol rings of these three ligands are oriented similarly and away from the 515–535 region, then it is the D-ring end of estradiol and the corresponding narrower region of hexestrol and the wider region of P1496 that are likely to be in contact with this region of the ER.

The pattern of residues that affect the transcriptional potency of TOT is rather unique. The interaction of TOT with the middle of the helical region is limited. In particular, its lack of interaction with His\textsuperscript{524} suggests that in lacking a second hydroxyl group, TOT is indifferent to the hydrogen bonding characteristics of the residue at this site. On the other hand, TOT is the only ligand whose transcriptional potency is affected by residues near the N terminus of the examined region (Arg\textsuperscript{515} and His\textsuperscript{516}), suggesting that its side chain may extend to be in contact with this region of the receptor.

Altogether, the comparison of ligand structure with the pattern of residues where mutation affects their transcriptional potency suggests that selected residues in the 515–535 region of the receptor are in contact with a portion of these ligands that corresponds to the D-ring end of estradiol. Thus, a rough comparison can be made between the orientation of these estrogens in ER and the RA and T\textsubscript{3} ligands in RAR and TR, respectively. It is the D-ring end of estradiol that corresponds to the apolar end of RA and the phenol of T\textsubscript{3} that is in contact with the helix-11 region of these nuclear receptors. The A-ring or phenolic portion of the estrogen ligands, which corresponds to the polar end of the RA and T\textsubscript{3} ligands, is directed away from helix-11.

It is well recognized now that the ER and related nuclear receptors show transcriptional activity that is modulated by the nature of the particular gene promoter and the cellular background, consistent with ligand-receptor interaction with
cell- and promoter-specific factors and transcriptional coactivators (5, 34). The interplay between the shape of an ER ligand and the residues that support its binding to ER, as studied here, may potentially underlie the selective actions of different ER ligands in various target cells and promoter contexts.

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