Determinants of Ligand Specificity of Estrogen Receptor-α: Estrogen versus Androgen Discrimination*

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We have been interested in understanding how the estrogen receptor (ER) binds estrogens and discriminates between different classes of steroids with closely related structures. Using insights from our prior studies on ER and from sequence comparisons of steroid receptors, we identified three residues in the hormone-binding domain of the human ER, Leu345, Thr347, and Glu353 that we considered were likely to be involved in steroid A-ring recognition and therefore estrogen versus androgen discrimination. We then tested the effect on ER activity of mutating these ER residues to the corresponding androgen receptor residues. Specifically, we examined the ability of the mutant receptors to bind and be activated by 17β-estradiol and three different androgens. No change in receptor activity was observed with the T347N mutation, while the L345S mutation greatly reduced ER activity in response to all ligands. Interestingly, the E353Q substitution behaved as expected, causing a 9-fold reduction in the transactivation potency of estradiol and a concomitant 10–140-fold increase in the transactivation potency of different androgens. These reciprocal changes in the transcriptional effectiveness of estrogens and androgens correlated with a decreased affinity of the E353Q ER for estradiol binding and an increased affinity for androgen binding. Therefore, amino acid Glu353 appears to be playing a significant role in binding the A-ring phenolic group of estradiol and in receptor discrimination between estrogens and the most closely structurally related steroids, androgens. Based on this data and our earlier observations, we propose a model for the orientation of ligand within the binding pocket of ER in which the A-ring 3-phenol of estradiol is hydrogen bonded to Glu353 in helix-3 and the 17β-hydroxyl of estradiol is hydrogen bonded to His354 in helix-11. Our findings with estrogen and androgen suggest that this orientation of the steroid in the ligand-binding pocket, with the A-ring in contact with helix-3 and the D-ring in contact with helix-11 residues, is likely to be general for all of the steroid hormone receptors.

The distinctive biological effects of the different classes of steroid hormones were recognized early in this century, when the basic physiology of the endocrine system was being elucidated. Despite their overall structural similarity, each class of steroid hormones does have characteristic structural features. In this regard, the estrogens (C18 steroids) have a characteristic aromatic A-ring with a phenolic hydroxyl at C-3, which uniquely distinguishes them from the other four steroid classes, androgens, progestins, glucocorticoids, and mineralocorticoids, all of which are 3-keto steroids. Among the latter, the structural differences are principally in their C- and D-rings. The androgens (C19 steroids) are identical to the estrogens in this region, whereas the progestins and corticosteroids (C21 steroids) all have distinctive hydroxylation and/or oxidation patterns in their C- and D-rings.

The steroid hormones are now known to act through specific receptors that belong to the nuclear hormone receptor superfamily, many of whose members have been cloned within the last decade (1–5). These complex proteins have separate DNA-binding and hormone-binding domains and act as ligand-dependent transcription factors. Despite their similar structures, each of the ligands acts specifically through its own receptor in vivo. The specificity of the estrogen receptors for estrogens is particularly strong. Nearly all good ligands are A-ring phenols, whereas those lacking this group, including androgens, corticosteroids, and progestins, are extremely poor ligands (6). Conversely, among receptors for the 3-ketosteroids, less specificity in binding between the four classes of ketosteroids is observed (7). Direct sequence comparison of the ligand-binding domains of the steroid receptors now reveals that the 3-ketosteroid receptors are more closely related to each other than they are to the estrogen receptor (8).

We have been intrigued by the structural features of the estrogen receptor, as well as the other steroid receptors, that underlie their ability to discriminate among ligands from the different steroid hormone classes. To gain a better understanding of the interactions between receptor and hormone, we have taken a structure/sequence-guided mutagenesis approach to study how estrogen receptor-α (ER)1 discriminates between estrogens and androgens. Because androgens differ from estrogens only in the A-ring region (Fig. 1), we felt a comparison between these classes of ligands would be most direct and informative. We focussed our attention to defining the regions of ER most likely to discriminate between estrogens and androgens, that is, A-ring binding.

In this study, we selected three residues in ER that we considered likely to determine ligand A-ring specificity, Leu345, Thr347, and Glu353, and used site-specific mutagenesis to replace them with the corresponding residues in the androgen

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1 The abbreviations used are: ER, estrogen receptor; AR, androgen receptor; CAT, chloromphenicol acetyltransferase; CMV, cytomegalovirus; DHT, 5α-dihydrotestosterone; E₁, 17β-estradiol; ERE, estrogen response element; HBD, hormone-binding domain; norT, 19-nortestosterone; T, testosterone; THC, tetrahydrocrysene.

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Estrogen Receptor Steroid Hormone Discrimination

receptor (AR). We then investigated the ability of the mutant receptors to bind and be activated by estradiol and a set of three androgens. The greatest shift in specificity occurred with the E353Q substitution, which caused a 9-fold reduction in interaction with estradiol and a 10–140-fold increase in interaction with different androgens. Mutations at the other positions, as well as combinations of these mutations, had less pronounced effects on ligand discrimination. We conclude that the interaction of the C-3 phenolic hydroxyl group of estradiol with its receptors in this orientation.

EXPERIMENTAL PROCEDURES

Reagents—The plasmids (ERE;pS2-CAT (9), pCMV5-hER (10), pCMVβ (CLONTECH, Palo Alto, CA), pCH110 (Pharmacia Biotech, Piscataway, NJ), and pTZ19R (11) have been described. Plasmid DNAs used for transfection were purified either by CsCl gradient centrifugation (12) or by Qiagen plasmid preparation kit according to the manufacturer's instructions (Qiagen, Chatsworth, CA). Ligand, 17β-estradiol, testosterone, 5α-dihydrotestosterone, and 19-nortestosterone were obtained from Sigma. Restriction enzymes and DNA polymerase were purchased from New England Biolabs (Beverly, MA). Oligonucleotides were purchased from Life Technologies, Inc. (Gaithersburg, MD). Cell culture media, calf serum, and other reagents for cell culture were purchased from Life Technologies, Inc. and Sigma. For Western blot analysis, nitrocellulose membrane was obtained from Millipore (Marlborough, MA), anti-ER H226 antibody was kindly provided by Dr. Geoffrey Greene (University of Chicago), and rabbit anti–rat IgG was acquired on a Spex Fluorolog 2 (model IIIC) instrument using Spec DM3000 software. The data was collected at 4 °C, in a ratio mode, using photomultiplier correction and 2.5-mm slits. Excitation was 379 nm. Unliganded ER fluorescence was subtracted as background. The ER hormone-binding domains were expressed in Escherichia coli in a pET11b vector, using standard methods (Novagen (12)) and purified by batchwise adsorption onto a nickel resin, according to the manufacturer's suggestions (Qiagen). HBDe were purified to 6 nM in TG buffer and incubated for 60 min at 0 °C with 2 nM tritiated hormone (THC-nitrile) (24, 25), or no ligand for background. The free THC-nitrile was not removed from these conditions nearly all of the ligand is bound. Spectra were measured at several ratios of ER to THC-nitrile to establish that free ligand was not affecting the position of the fluorescence peak.

RESULTS

Selection of Sites in the Estrogen Receptor Likely to Determine Ligand A-Ring Specificity—In our recent studies of ligand-receptor contact sites (14, 26) we used alanine scanning mutagenesis over a 21-residue region of ER to identify likely ER domain and contact points. This region of the ER hormone-binding domain (HBD) is now thought, based on comparisons with the retinoic acid receptor-retinoic acid (27) and the thyroid hormone receptor-triiodothyronine (28) HBD crystal structures, to form a portion of helix-11 and the loop between helix-11 and -12. When we investigated the footprint of ligand contacts for four ER ligands that had very similar A-ring structures but differed substantially in their D-ring regions, we found that each ligand had a unique, characteristic footprint (26). We therefore concluded that helix-11 in ER formed a portion of the ligand-hinging pocket that accommodated the D-ring region of estradiol and related estrogens. Interestingly, sequence similarity in the helix-11 region of different steroid receptors is low, consistent with a requirement to accommodate hormones that have different and distinctive patterns of D-ring polar functions, suggesting that all the steroids may bind to their receptors in this orientation.

If the steroids bind with their D-rings near helix-11 and then orient the rest of their structure along the same axis as does the
ligand in the retinoic acid receptor-retinoic acid and thyroid hormone receptor-triiodothyronine crystal structures (27, 28), then the A-rings would extend toward and contact portions of helix-3 and -5. We searched the sequences of the steroid receptors over these regions for potential A-ring C-3 recognition/discrimination sites, amino acid differences likely to reflect the characteristic functional difference between binding a 3-phenol versus a 3-keto steroid (Fig. 1). Because the steroid hormone receptor sequences in helix-5 are highly conserved, we did not select any sites in that helix.

We chose to investigate three sites in helix-3 which are highlighted in the sequence alignments shown in Fig. 2. These sites appear to represent positions where the ER residue is of a different character (presumably selected to favor interaction with a 3-phenol) from that at the corresponding position in the sequence the other four steroid receptors (presumably selected to favor interaction with a 3-keto group). Leucine 345 in ER is replaced by a smaller, more polar residue (serine or threonine) in the other receptors. Similarly, threonine 347 in ER is replaced by an asparagine, and glutamate 353 in ER is replaced by a glutamine. At the other positions shown in Fig. 2, the residues in ER versus the other four receptors are either identical or highly homologous, or they show no systematic change between the receptor classes.

The glutamate versus glutamine substitution at position 353, in particular, represents the type of change expected for recognizing the functional group difference between estrogens and 3-ketosteroids at the ligand C-3 position. The glutamate of ER, a strong hydrogen bond acceptor, would pair well with the strong hydrogen bond donor phenol of estrogens. Conversely, the good hydrogen bond donor glutamine is matched with the good hydrogen bond acceptor 3-keto function in the ligands for androgen, progesterone, glucocorticoid, and mineralocorticoid receptors (AR, progesterone receptor, glucocorticoid receptor, mineralocorticoid receptor, Fig. 3).

Transcriptional Response of Estrogen Receptor Mutants with 17β-Estradiol and Androgens—Using site-directed mutagenesis of the ER cDNA, we mutated Leu345, Thr347, and Glu353 of the HBD to match the AR amino acid sequence; serine, asparagine, and glutamine, respectively. We generated three single amino acid substitutions, as well as a double and a triple mutation. We then tested the ability of the mutant ERs to activate transcription in response to E2, as well as three androgens, 5α-dihydrotestosterone (DHT), testosterone (T), and 19-nortestosterone (norT) (Fig. 1). These results are shown in Figs. 4 and 5, and in Table I. Transcriptional activity was measured in ER-negative human breast cancer MDA-MB-231 cells transfected with an ER expression vector and an estrogen response element (ERE)-containing reporter gene construct,
CAT reporter, pCMV

3 concentrations (activities were normalized to before preparation of extracts. CAT activity of the wild type and mutant ERs.

The T347N mutation had no effect on ER's ability to be activated either by estrogens or androgens, and the transactivation of these different ligands to induce transcriptional response to E2 was also diminished. Likewise, no activation by the ligands, activated wild type receptor to the same maximal level. 

Conversely, the L345S mutation elicited little to no change in androgen responsiveness compared with wild type. The triple mutant, with changes at amino acids 345, 347, and 353 (Fig. 4D, denoted LTE-SNQ ER) also showed poor response to E2 and the same response as wild type ER to androgens.

In Fig. 5, the data are arranged to show how the receptors respond to each of the ligands tested. Response to estradiol was best with the wild type and T347N ERs, followed by E353Q ER, and then L345S, with the double and triple mutants showing a 10-fold reduced transactivation effectiveness of estradiol (Fig. 5A). Response to the three androgens (Fig. 5, panels B-D) was best with the E353Q ER. The androgens were approximately 20-fold less effective with the two other single or the double mutant ERs. Of note, the L345S receptor showed no stimulation by any of the androgens, yet was moderately responsive to estradiol (Fig. 4C).

Western Blot Analyses of Mutant ER Protein Levels—To determine whether protein stability and/or expression levels contributed to any of the differences in observed transactivation, respectively.

**TABLE I**

| Estrogen receptor | Ligand transactivation potency (relative EC_{50}) |
|-------------------|-----------------------------------------------|
|                   | E2  | T    | DHT  | 19-norT |
| Wild type         | 100 | 0.0014 | 0.0032 | 0.0055 |
| E353Q             | 11  | 0.20 (143×↑) | 0.079 (25×↑) | 0.069 (13×↑) |
| T347N             | 139 | 0.0011 | 0.0031 | 0.0046 |
| L345S             | 0.55 | BD' | BD  | BD |
| TE-NQ             | 0.0095 | 0.0021 | 0.0017 | 0.0037 |
| LTE-SNQ           | 0.0073 | 0.0011 | 0.0017 | 0.0021 |

- Relative EC_{50} = (EC_{50}^{E2} (wt ER)/EC_{50}^{endpoint})/100; EC_{50}^{E2} (wt ER) = 0.11 nM.
- Numbers in parentheses are fold change in ligand potency in E353Q ER versus wild type ER.
- BD, below detection.
- Double mutant: T347N,E353Q.
- Triple mutant: L345S,T347N,E353Q.

**FIG. 4.** Transactivation profiles for wild type (wt) estrogen receptor and mutant ERs in response to estradiol and three androgens. MDA-MB-231 cells were cotransfected with (ERE)_2-pS2-CAT reporter, pCMVβ internal control plasmid, and the indicated ER expression plasmid. Transfected cells were then treated with the indicated ligand for 24 h before preparation of extracts. CAT activities were normalized to β-galactosidase activity and are expressed relative to the wild type ER activity with 10^{-12} M estradiol (100–140-fold stimulation in different experiments), which is set at 100%. The values represent the mean and S.D. from two or more experiments. For some values, error bars are too small to be seen. In panel A, data for wild type ER is shown with filled symbols and dashed lines, and data for T347N ER with open symbols and solid lines.
we performed Western blot analyses. The ERs were expressed in COS cells, and extracts were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with antibody specific for the ER. Each of the mutant receptors was expressed at levels very similar to that of wild type ER (data not shown). Similar results were obtained when the receptors were expressed in MDA-MB-231 cells or in the presence of 10⁻¹⁰ M E₂. The level of ER-bound E₂ was determined, and Scatchard analyses were performed. The Kᵣ determined for wild type receptor was 0.055 ± 0.027 nM (n = 4); E353Q ER had a Kᵣ of 0.16 ± 0.08 nM (n = 4) (data not shown).

In the four experiments, E353Q ER had a 3.1 ± 1.5-fold lower affinity for E₂ than wild type ER.

Relative binding affinities for the androgens were also determined using a competitive radiometric binding assay. For these experiments, cell extracts containing expressed wild type ER or E353Q ER were incubated with tritiated E₂ plus increasing concentrations of unlabeled competitor (10⁻¹⁰-10⁻⁵ M E₂, DHT, norT, or T). The results, presented in Table II, show very low relative binding affinities (0.052, 0.0014, and 0.00035% for DHT, norT, and T, respectively) for the wild type ER, but very significantly increased affinities of the androgens for the mutant E353Q ER (19.6, 4.39, and 3.03% for DHT, norT, and T, respectively) for the wild type ER, but very significantly increased affinities of the androgens for the mutant E353Q ER (19.6, 4.39, and 3.03% for DHT, norT, and T, respectively). Thus, the improved transactivation ability of these androgens with the E353Q ER can be explained to a large degree by the greatly improved affinity of these androgen ligands for this E353Q estrogen receptor.

Fluorescence Probing of Ligand Receptor Interaction—We have developed a series of non-steroidal ligands for the ER, the tetrahydrochrysenes (THCs, see Fig. 1) (24), that have excellent fluorescent properties and have been used to assay the binding of ligands to ER and the distribution of the receptor in cells (25, 29). An unusual characteristic of these fluorescent estrogens is that they emit at longer wavelengths when bound
to ER than in aqueous solution. We ascribed this receptorinduced red shift to a specific interaction between the THC and residues in the ER ligand pocket that stabilize the excited state of the fluorophore (25). This stabilization could be achieved through a polar or charge interaction between the phenolic function of the THCs and a nearby residue in ER, such as Glu353.

We therefore investigated this possibility by comparing the fluorescence emission spectra of the tetrahydrochrysene-nitrile (THC-nitrile, see Fig. 1) when bound to wild type ER versus E353Q ER. To perform these experiments, large quantities of the HBDs of these two receptors were expressed as His-tagged proteins in *E. coli* and were purified over a nickel column. As shown in Fig. 6, the THC-nitrile when bound to wild type ER shows a fluorescence emission of 510 nm, close to that reported by us with uterine ER preparations (25, 29). In contrast, the emission of the THC-nitrile when bound to E353Q shifted even further to the red, emitting at 567 nm. Thus, the mutational change of glutamate to glutamine at position 353 in ER has a very significant effect on the fluorescence of the THC-nitrile, providing further support to the direct interaction of this site with the phenolic function of estrogens.

**DISCUSSION**

Glu353 in the Estrogen Receptor Plays an Important Role in Binding the A-Ring Phenolic Hydroxyl Group of Estradiol and in Discrimination between Estrogens and Androgens—We have used information from steroid receptor sequence analysis, comparison with crystal structures of related proteins, and previous mutational studies on ER done by us to select residues in the ligand-binding domain of the ER that appeared likely to be in contact with the estradiol A-ring phenolic hydroxyl group. By investigating amino acid substitutions made at these positions, we identified amino acid Glu353 as playing a significant role in discriminating between estrogens and the most closely structurally related steroids, androgens. The recognition of estradiol at the Glu353 site is likely to occur by the more favorable interaction that the anionic carboxylate in the glutamate residue has with the phenolic hydroxyl of estradiol, than with the 3-keto group of other steroids from the other hormone classes (cf. Fig. 3).

We demonstrated that mutation of the glutamate residue at position 353 in the ER to the glutamine found in AR, progesterone receptor, glucocorticoid receptor, and mineralocorticoid receptor, reduced the potency of estradiol in inducing ER transactivation, while at the same time increasing the potency of several androgens. The dramatic effect that this single mutation has both in terms of the “loss of function” toward estrogens and the “gain of function” toward androgens is striking, and indicates that the role of this site in discriminating between these classes of hormonal steroids is significant. By contrast, mutations at two other positions, Thr347 and Leu345, did not have similar effects. The T347N change had no observable affect on ER activation by either estradiol or by androgens and is therefore unlikely to play a role in ligand discrimination. L345S, however, reduced ability of both estradiol and androgens to stimulate ER activity. This mutation, while not affecting overall stability of the protein as demonstrated by Western blot analysis, may be altering the overall conformation of the receptor, rendering ER unable to bind ligand well or to activate transcription when ligand is bound. The placement of Leu345 and Thr347 relative to Glu353 on an o-helical wheel makes it unlikely that all three residues would contact ligand.

Despite the marked reciprocal shift in the relative potency and affinity of estradiol versus androgens that results from the E353Q mutation, the residue at this site alone does not fully define the estrogen versus androgen hormonal specificity of these receptors. E353Q ER still binds androgens less well than estrogens, although with much less of a difference than wild type ER. Thus, it is clear that amino acids at other locations are important contributors to estrogen/androgen ligand discrimination by these receptors.

Functional mapping approaches have also been used to define ligand contact sites in other receptors. Using the fact that chick progesterone receptor fails to bind the antiprogestin RU486, whereas human progesterone receptor binds it well, Garcia et al. (30) used a segmental chimera approach followed by single site substitutions to identify a residue in helix-3 (at a position that would correspond to Ala350 in ER) as the receptor site most likely to be close to substituents at the 11β-position of a progesterone ligand. More recently, Vivat et al. (31) used a similar segmental chimera/mutational approach to identify some regions in AR and progesterone receptor that are important in discrimination between ligands from the androgen and progesterin hormonal classes.

**Comparison between the Transcription Activation Potency and the Binding Affinity of Estrogens and Androgens with ER Mutants**—The transcription assays show that the E353Q mutation affects the relative potency of estradiol and the three androgens, but not their efficacy, as all ligands were able to induce maximal levels of transcriptional activity at sufficiently high ligand concentrations. However, the issue of changes in ligand transactivation potency versus ligand binding affinity is interesting and complex. The E353Q mutation in ER results in a 9-fold decrease in the potency of E2 as a transcriptional activator, but only a 3-fold decrease in E2 binding affinity is observed. In our earlier study of alanine mutants in helix-11 of the ER-HBD (14), we found that there was a good correlation between the shift in E2 transactivation potency and binding affinity in the three mutants for which we could measure E2 binding affinity. In the current study of helix-11 alanine mutants, we noticed that a good correlation between the shift in E2 transactivation potency and binding affinity existed only for mutations of Thr347 and Leu345. In contrast, a related mutation in the same region of the estrogen receptor (32) did not correlate well with the binding affinity of estradiol. In summary, the binding affinity of estradiol to the ER is decreased upon mutation of residues Thr347 and Leu345, but the binding affinity of estradiol to the progesterone receptor is increased upon mutation of these residues. The results of the current study suggest that a single point mutation in the ER-HBD can cause a greater decrease in transactivation potency than in binding affinity for one endogenous of the non-steroidal estrogen, indenestril B. This quantitative difference between the binding affinity versus transcriptional potency might potentially reflect perturbations in the coupling between the ligand-receptor complex and co-activator proteins.
such as SRC-1 that mediate the transcriptional response (5).

In this paper, we also examined the transcriptional potencies and binding affinities of several androgens with wild type and E353Q ERs (Table II). In competitive binding assays, using \(^{3}H\) E2 as tracer, the androgens showed greatly increased binding to E353Q ER; even when the 3-fold difference in E2 binding to the E353Q ER is taken into consideration, the increases are 130–3,000-fold.

It is of note that the relative affinity of the three androgens for the E353Q ER (DHT > norT > T) is the same as their relative affinity for AR itself. Relative to the androgen methyltrienolone (R1881), their affinities for AR are: DHT, 61 ± 17% (n = 3); norT, 31 ± 2% (n = 2); and T, 6.7 ± 14.4% (n = 6).2 The second item of note are differences in the relative binding affinity and transactivation potencies of the three androgens. This comparison is made in the columns marked “Index” in Table II, which shows the ratio of binding affinity to transactivation potency. By this index, the potency of DHT with both wild type ER and E353Q ER is much less than expected from its index; the index values for T and norT are much smaller. Although these differences could be due to altered coupling between co-activators and the ligand-receptor complex (5), the fact that DHT is most affected suggests another explanation.

The binding assays are done with cell extracts under dilute protein concentrations in the absence of serum, whereas the transactivation assays are done with cells in 5% calf serum. Calf serum is known to contain a protein related to sex hormone-binding globulin (33). Although the binding specificity of the bovine protein has not been characterized in detail, human sex-hormone-binding globulin binds DHT 20-fold better than E2. T is bound only 4-fold better, and norT 3-fold less well then E2.2 Thus, greater binding of DHT by serum components in the transactivation assay could account for its reduced transactivation potency relative to receptor binding affinity.

Functional Mapping of Ligand-Receptor Contact Sites: A Model for the Orientation of Estradiol and Other Steroid Hormones in the Ligand Binding Pocket of Their Receptors—By combining our identification in this study of Glu\(^{353}\) as the likely A-ring phenol-binding site of estrogens in ER, with our earlier conclusion that the D-ring portion of the ligand is in contact with helix-11 (26), we have defined the basic axis and orientation of the ligand within the binding pocket of the hormone-binding domain of ER. The D-ring of E2 is in contact with helix-11, with the 17β-hydroxyl group most likely hydrogen bonded to His\(^{225}\) (26), and the A-ring is projected toward helix-3, with the 3-phenolic hydroxyl hydrogen bonded to Glu\(^{353}\). Our findings further suggest that the other steroid hormones will adopt the same A-ring/helix-3–D-ring/helix-11 orientation. It is of note that this orientation of steroid ligands in the binding pocket of steroid receptors is opposite of that proposed by others on the basis of homology models built from the non-steroid-receptor retinoic acid receptor-retinoic acid crystal structure (34). Final verification of the ligand orientation within the ER and other steroid receptors will need to await reports of the crystal structures of these hormone-receptor complexes.

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