Directed Evolution of Human Estrogen Receptor Variants with Significantly Enhanced Androgen Specificity and Affinity*\[S\]

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Human estrogen receptor α (hERα) and human androgen receptor exhibit exquisite ligand specificity, which underlies their remarkable ability to effect ligand-regulated gene transcription in a highly distinctive and specific manner. Here we used a directed evolution approach to create hERα variants with enhanced androgen specificity and affinity with the goal to better understand the molecular basis of ER ligand specificity and the evolutionary mechanism of nuclear receptors. We developed a sensitive yeast two-hybrid system to screen for hERα variants with increased transactivation potency toward testosterone. After two rounds of directed evolution, we identified five hERα variants with dramatically improved transactivation potency toward testosterone in both yeast and mammalian cells. These variants showed up to 7,600-fold improvement in the binding affinity for testosterone and only slightly reduced affinity toward 17β-estradiol. Detailed analysis of these evolved variants and a few site-directed mutants generated de novo led to several unexpected findings including the following. 1) Only two beneficial mutations were needed to create hERα variants with near nanomolar affinity for testosterone. 2) Some beneficial mutations were synergistic, context-dependent, or non-additive. 3) Of the five identified beneficial mutations, four of them were not in the ER ligand binding pocket and yet exerted important action on ligand specificity. 4) The single ligand-contacting mutation E353Q plays a dominant role in discriminating androgens and estrogens. These results, viewed in conjunction with the ligand exploitation model of nuclear receptor evolution, suggest that the mutation E353Q may represent a key event in the evolution of androgen receptors from an ancestral estrogen receptor and that ligand promiscuity may play an important role in the creation of new nuclear receptors via divergent evolution.

The estrogen receptor (ER)† and androgen receptor (AR) belong to the steroid hormone nuclear receptor superfamily

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† The abbreviations used are: ER, estrogen receptor; AR, androgen receptor; DHT, 5α-dihydrotestosterone; ERE, estrogen response element; EC50, half-maximal effect concentration; E2, 17β-estradiol; HEC-1, human endometrial cancer; LBD, ligand binding domain; MOE, molecular operating environment; RBA, relative binding affinity; SRC-1, steroid receptor coactivator 1; T, testosterone; Pg, Progesterone; h, human.

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intrigued by the structural features of hER/H9251 and hAR that underlie their ability to discriminate E\textsubscript{2} from testosterone. Prior site-directed mutagenesis studies have indicated that the hER/H9251 residue Glu-353 plays a significant role in discriminating estrogens from androgens (9). However, that single mutation alone cannot account for the observed difference in ligand affinity and specificity between the ER and AR. To gain a further understanding of the interactions between the receptor and ligand, we have taken a new approach, directed evolution. Directed evolution is a powerful tool for engineering proteins with improved functions, such as solubility, stability, affinity, and activity (10, 11). Here we report the application of directed evolution to engineer hER/H9251 variants with up to 7,600-fold improvement in the binding affinity toward testosterone. Strikingly, these evolved hER/H9251 variants exhibited only slightly reduced binding affinity (1.5–3-fold) to \textit{E}_2 and contained only two or three amino acid substitutions in the LBD. Further biochemical and structural analysis of these evolved ER variants provided new insights into the molecular basis of ER ligand specificity and the evolutionary mechanism of steroid receptors.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Cell culture media were purchased from Invitrogen. Calf serum was obtained from HyClone Laboratories, Inc. (Logan, UT), and fetal calf serum was purchased from Atlanta Biologicals (Atlanta, GA). The luciferase assay system was from Promega Corp. (Madison, WI). The 17\textbeta\textsubscript{-}estradiol, testosterone, dihydrotestosterone, and isopropyl\textbeta\textsubscript{-}D-thiogalactopyranoside were from Sigma. All restriction enzymes and DNA-modifying enzymes were obtained from New England BioLabs (Beverly, MA). Yeast strain YRG-2 (\textit{Mat a ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3112 gal4-542 gal80-538 lys2:UAS\textit{GAL1}-TATA\textit{GAL1}-HIS3 URA3:UAS\textit{GAL1} 17mers(x3)-TATA\textit{GAL1}-lacZ) were from Stratagene (La Jolla, CA). Taq DNA polymerase was from Promega Corp. \textit{\beta\textsubscript{3}}\textit{HIE} \textsubscript{5} (50 Ci/mmoll) was obtained from Amer sham Biosciences. QIAprep spin plasmid miniprep kit, QIAEX II gel purification kit, and QIAquick PCR purification kit were purchased from Qiagen (Valencia, CA). Various

![Fig. 1. A, sequence alignment between hERα LBD and hAR LBD. Residues not included in the crystal structures are signified by lowercase letters. Residues shown in italicized lowercase letters are hERα F domain residues. The residue number applies to the residue directly above or below the first digit. Identical residues between hERα LBD and hAR LBD are highlighted with gray shading, and ligand-contacting residues are outlined in boxes (van der Waals cut-off distance, 4.0 Å). Mutations are shown in boldface. The 12 α-helices are underlined and numbered. B, chemical structures of \textit{E}_2, T, DHT, and Pg. H2–H12, helices 2–12.](image)
hERa Variants with Androgen-like Specificity

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oligonucleotide primers were obtained from Integrated DNA Technolo-
gies (Coralville, IA).

Plasmid Construction—pCMV5-ERE plasmid containing the wild
type full-length hER α cDNA (12) was first digested with Eagl and
BamHI and then treated with T4 DNA polymerase to form blunt ends
at both termini followed by ligation into the EcoRI site (also treated
with T4 DNA polymerase) of pBD-GAL4-Cam vector (Stratagene)
to form pBD-GAL4 hER α containing hER α amino acids 312–595.
pGAD424 SRC-1 containing the full-length SRC-1 was constructed as
described elsewhere (13). Mammalian cell reporter plasmid (ERE)_2-
ERE that contained two tandem repeats of the ERE sequence was
constructed as the pBD-GAL4 hER α reporter. The resulting mutagenized
LBD fragments were then co-transformed with pBD-GAL4 hER α and
to determine their EC50 values. In the 96-well plate assay, yeast YRG2
plates were set up with buffer G (50 mM Tris buffer, pH 8.0, 10%
glycerol, and 10 mM β-mercaptoethanol) at 10 μg/mL of wet cells, and
the absorbance at 600 nm was measured using a microplate reader
(Eclipse 800, Perkin-Elmer, Boston, MA). Results were considered to be
positive if they were greater than the mean plus two standard errors
of the controls. All experiments were performed in triplicate.

DNA Shuffling and DNA Sequencing—DNA shuffling of the second
round hER α variants was carried out as described elsewhere (17). DNA
sequencing of the evolved hER α variants was carried out as described
in Ref. 18.

Cell Culture and Transfection—Cell culture and transfection were
performed as described elsewhere (19).

Hormone Binding Assays—E. coli BL21(DE3) cells transformed with
the wild type or mutant hER α LBD fragments encoding residues 312–595 were grown at 37 °C until A600 = 0.6. Protein expression was then induced with 0.5 mM isopropl-β-D-
thiogalactopyranoside at 25 °C for 5–7 h. Cells were harvested by
centrifugation, resuspended in buffer B (50 mM Tris buffer, pH 8.0, 10%
glycerol, and 10 mM β-mercaptoethanol) at 10 μg/mL of wet cells, and
then adjusted to the standard protocol suggested by the manufacturer.
The cell debris were separated from the superna-
tants by centrifugation for 30 min at 10,000 × g. The resulting sup-
ernants were used in all hormone binding assays.

The equilibrium ligand binding assay was performed essentially as
described elsewhere (19) except that E. coli cell extracts rather than
plasmids were used as the template for the overlap extension PCR,
Library Creation and Screening—Error-prone PCR was used to gen-
erate a library of mutated LBD fragments consisting of hER α
amino acids 312–595 using pBD-GAL4 hER α as a template for the
ERE and ERLBDCam5F and ERLBDCam3R (5'-tca gac tgt gcc gag acc-3')
primers according to the protocol described elsewhere (15). The
resulting mutated LBD fragments were then co-transformed with the
pBD-GAL4 hER α vector that was digested with BglII and BsaI to
form 94-bp gaps into yeast YRG2 cells pretransformed with the
pGAD424 SRC-1 plasmid. Yeast transformation was carried out using
the lithium acetate/single-stranded carrier DNA/polycethylene glycol
protocol (16).

For library screening, a two-tiered strategy consisting of an agar
plate-based selection followed by a 96-well plate-based screening was
used. In the selection method, the mutated LBD fragments were co-
transformed with pBD-GAL4 hER α as a template for the
ERE and ERLBDCam5F and ERLBDCam3R (5’-tca gac tgt gcc gag acc-3′)
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pGAD424 SRC-1 plasmid. Yeast transformation was carried out using
the lithium acetate/single-stranded carrier DNA/polycethylene glycol
protocol (16).
RESULTS

Library Screening Using Yeast Two-hybrid System—To isolate human ERα variants with altered transcriptional activation (transactivation) activity, we developed an efficient and sensitive high throughput screening method based on the yeast two-hybrid system. In this method, the cDNA encoding hERα amino acids 312–595 containing most of the LBD domain (hERα amino acids 303–553) and the F domain (hERα amino acids 554–595) was fused to the gene encoding the GAL4 DNA binding domain in plasmid pBD-GAL4-Cam (Stratagene) to form the “bait plasmid” pBD-GAL4 hERα, and the gene encoding human SRC-1 was fused to the gene encoding the GAL4 activation domain in plasmid pGAD424 (Clontech) to form the “prey plasmid” pGAD424 SRC-1.

Both plasmids were transformed and expressed in S. cerevisiae YRG2, which contains a GAL4-regulated HIS3 reporter construct on its chromosome. The HIS3 reporter provides strong nutritional selection (only cells expressing HIS3 gene product, which is an essential enzyme in the histidine biosynthetic pathway, can grow in a minimal medium lacking histidine). In the presence of agonistic ligands, the LBD undergoes a conformational change and binds to SRC-1, which brings the GAL4 DNA binding domain and the GAL4 activation domain in proximity, thus activating the transcription of the reporter gene. In general, the cell growth rate is proportional to the strength of the ligand-receptor interaction. In the absence of agonistic ligands, no transcription of reporter genes will occur. The functional interaction of ERα LBD with the coactivator SRC-1 is critical for effecting transcription in mammalian cells (5).

We have validated this yeast two-hybrid-based selection/screening method using the wild type hERα LBD in yeast cells. All experiments were done in the 96-well plates where A500 (cell density) was measured. First, it was shown that cells bearing plasmids pBD-GAL4 hERα and pGAD424 SRC-1 responded to E2 at a subnanomolar concentration, while cells bearing plasmid pBD-GAL4 ERα alone had no response to E2 up to a micromolar concentration, indicating that the cell growth assay is tripartite (Fig. 2A). Second, it was found that the ability of cells bearing plasmids pBD-GAL4 hERα and pGAD424 SRC-1 to activate transcription in response to a ligand generally correlated with the RBA of the ligand (E2 (100) > 17α-estradiol (58) > 2-0H-estradiol (7) > testosterone (<0.01) > progesterone (<0.001), the RBA values (in parentheses) were taken from Ref. 21) with greater response seen with ligands with higher RBAs (Fig. 2B). These results indicate this selection/screening method is very sensitive and specific. It should be noted that the half-maximal effect concentration (EC50, the ligand concentration that causes a half-maximal response) of the wild type hERα LBD for E2 was estimated to be 0.6 nM, which is in good agreement with the reported value of the full-length hERα for E2 binding (12).

For library screening, we used a two-tiered strategy consisting of an agar plate-based selection followed by a 96-well plate-based screening. In the selection method, the mutagenized hERα LBD variants were co-transformed with pGAD424 SRC-1 into S. cerevisiae YRG2 and plated on an agar plate containing minimum medium lacking tryptophan, leucine, and histidine and supplemented with a predefined testosterone concentration. To eliminate the false positives caused by mutations resulting in ligand-independent responses, the colonies that appeared on the agar plate were picked with toothpicks and restreaked onto two agar plates, one with testosterone and the other without testosterone. The colonies appearing on the agar plate with testosterone but not on the agar plate without testosterone were picked and assayed in the 96-well plate to determine their EC50 values. Moreover, to ensure that the improved EC50 value of a positive variant was plasmid-linked, the plasmid was rescued from the corresponding variant and transformed into fresh YRG-2 cells to confirm that the same EC50 value could be obtained.

Directed Evolution of hERαLBD Variants with Increased Response to Testosterone—In the first round of directed evolution, we used error-prone PCR to introduce random point mutations (one to two amino acid substitutions per gene on average) into the LBD fragment consisting of hERα amino acids 312–595. Transformed yeast cells bearing a library of hERα LBD variants (~60,000) were plated on minimum medium lacking tryptophan, leucine, and histidine and supplemented with 5 × 10⁻⁷ M testosterone. Cells bearing the wild type ERα LBD were used as a negative control. Fifty-three colonies appearing on the selection plates were picked and streaked on two plates, one with testosterone and the other without testosterone. Three ligand-dependent clones were identified and selected for a quantitative dose-response measurement in 96-well plates in which the cell densities were determined over a range of testosterone concentrations.

Two clones (T7 and T17) showed increased ligand sensitivity and were selected for the second round of directed evolution. Two independent libraries of mutants were created using error-prone PCR. Using the same screening strategy, ~120,000 clones result-
activities were normalized to response to testosterone in ER-negative human endometrial cells. hER200-fold more sensitive to testosterone than was the wild type data from yeast cells, the second round mutants were at least in the mammalian HEC-1 cells is quite different from those in ER mutants in yeast, the constellation of coregulators present with SRC-1 in a two-hybrid assay was used for the selection of compared with the fact that DHT is a more potent androgen than testosterone, both the wild type and mutant receptors showed at least 20-fold more sensitivity to testosterone than was the wild type hERα. The dose responses of most of the hERα variants to E2 and testosterone were, overall, in good agreement with their dose response in HEC-1 cells (Table I and Fig. 3).

To probe whether the mutations would result in a promiscuous receptor, we also evaluated two additional ligands, DHT and progesterone, for the wild type hERα and hERα variants (Table I and Fig. 3). In both yeast and HEC-1 cells, consistent with the fact that DHT is a more potent androgen than testosterone, both the wild type and mutant receptors showed at least 20-fold higher sensitivity to DHT than to testosterone. Moreover, the second round mutants were more sensitive to DHT than were the first round mutants. Interestingly, all the mutant receptors showed enhanced sensitivity to progesterone with the highest fold-improvement of >30 (T7-16 in yeast) compared with the wild type receptor, suggesting that the mutations have made the receptors more promiscuous.

It should be noted that differences observed in the transactivation activity of some mutants may reflect differences in their responsiveness to coregulators. Although ER interaction with SRC-1 in a two-hybrid assay was used for the selection of ER mutants in yeast, the constellation of coregulators present in the mammalian HEC-1 cells is quite different from those in yeast.

Identification of Functional Mutations in the Evolved hERαLBD Variants—To identify the molecular basis of altered ER ligand specificity, we sequenced all the evolved hERα LBDs to be true positives. Similarly ~110,000 clones resulting from T17 were grown with 10−8 M testosterone, and 20 clones were selected. Two of them (T17-2 and T17-4) were identified to be true positives. As shown in Table I, the EC50 values of the first round mutants for testosterone were increased more than 39-fold compared with that of the wild type ER LBD, and those of the second round mutants were increased another 6–20-fold (a total of more than 234–780-fold).

Transactivation Assay in Mammalian Cells—To determine whether the evolved ER mutants in yeast cells will behave similarly in mammalian cells, we evaluated the transactivation profiles of the full-length wild type hERα and hERα mutants in response to testosterone in ER-negative human endometrial cancer (HEC-1) cells. Briefly the genes encoding mutant hERα LBDs were subcloned into ER expression vector pCMV5 (12) to recreate full-length mutant hERα genes containing all the ER domains. HEC-1 cells were then transfected with luciferase reporter plasmid (ERE)2-pS2-Luc, the indicated ER expression plasmids, and pCMVβ internal control plasmid. Luciferase activities were normalized to β-galactosidase activity and were expressed relative to the wild type hERα activity with 10−8 M E2, which is set at 100%. Consistent with the dose-response data from yeast cells, the second round mutants were at least 200-fold more sensitive to testosterone than was the wild type hERα. The dose responses of most of the hERα variants to E2 and testosterone were, overall, in good agreement with their dose response in HEC-1 cells (Table I and Fig. 3).

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Functional Mutations Identified in the Evolved hERα Variants—To identify the molecular basis of altered ER ligand specificity, a series of single mutants, double mutants, and triple mutants were created using site-directed mutagenesis and assayed in yeast cells. As summarized in Table I and shown in Supplemental Fig. 7, relative to the wild type hERα, the single mutant F461I hERα showed a 4-fold reduced sensitivity to E2 and a similar sensitivity to testosterone, whereas the single mutant G390D hERα showed increased sensitivity to testosterone and a slightly reduced sensitivity to E2. The single mutant G442V hERα showed a similar sensitivity to E2 and at least a 2-fold increased sensitivity to testosterone. This mutant had a moderately elevated basal level response. In comparison, the single mutant L536H hERα showed a significantly elevated ligand-independent response and a slightly reduced sensitivity to E2. The single mutant A569T hERα also showed a similar sensitivity to testosterone and a slightly decreased sensitivity to E2. The double mutant G442V/F461I hERα showed sensitivities to both E2 and testosterone similar to the single mutant G442V hERα. Similarly, the triple mutants E353Q/G390D/G442V and E353Q/G390D/A569T hERα showed transcription potency to both ligands that is similar to that of the double mutant E353Q/G390D hERα. However, the triple mutant E353Q/G390D/L536H hERα had similar sensitivity to E2 but showed a very high basal level response, which made the estimation of EC50 value of testosterone impossible.

Hormone Binding Assay—To establish whether the improved transactivation potencies of these evolved hERα variants toward testosterone were the result of the corresponding changes in ligand binding affinities, we measured the binding affinities of these variants by both direct and competitive hormone binding studies. As summarized in Table I, the binding affinities of the wild type hERα and the evolved hERα variants for E2 (Kd) were determined by a direct hormone binding assay. The Kd values of the wild type hERα were 0.29 ± 0.02 nm, whereas the evolved hERα variants had increased Kd values toward E2, ranging from 0.44 to 3.53 nm (1.5–12-fold decreased affinity compared with the wild type hERα).

The RBAs of the wild type hERα and the evolved hERα variants for testosterone were also determined using a competitive hormone binding assay. On this RBA scale, the affinity of E2 for the wild type hERα is set at 100. The RBA of testosterone for the wild type hERα was less than 1, and the evolved variants had RBAs for testosterone ranging from 0.5 to 1.5. Based on these data, the binding affinities of the wild type hERα and the evolved hERα variants for testosterone (Kd) were calculated. As shown in Table I, all the second round hERα variants had close to nanomolar affinities to testosterone with the T7-18 showing the highest affinity (38 nM), which represents >7,600-fold improvement over that of the wild type hERα.

DISCUSSION

By using error-prone PCR and a yeast two-hybrid-based high throughput screening method, we isolated five hERα variants with significantly enhanced affinities for testosterone. Analysis of these evolved hERα variants using transactivation assays in yeast and mammalian cells, in vitro hormone binding assays, and molecular modeling provides some novel insights into the molecular basis of ER ligand specificity and the evolutionary mechanism of nuclear receptors.

Functional Mutations Identified in the Evolved hERα Variants

A total of seven hERα variants with enhanced response toward testosterone were isolated. These seven mutants contained six distinct point mutations, most of which are non-conservative changes. All these mutations were mapped into the crystal structure of the hERα-testosterone complex in our molecular modeling studies.
<table>
<thead>
<tr>
<th>Estrogen receptor</th>
<th>Yeast EC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HEC-1 EC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>K&lt;sub&gt;E2&lt;/sub&gt;&lt;sup&gt;a,b&lt;/sup&gt; (nM)</th>
<th>RBA&lt;sup&gt;c&lt;/sup&gt; (nM)</th>
<th>K&lt;sub&gt;T&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt; (nM)</th>
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<tr>
<td>Wild type</td>
<td>0.6 ± 0.14</td>
<td>&gt;10,000</td>
<td>0.032 ± 0.01</td>
<td>0.029 ± 0.02</td>
<td></td>
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<tr>
<td>E353Q/F&lt;sub&gt;461&lt;/sub&gt;I (T7)</td>
<td>9.9 ± 4.1</td>
<td>2.57 ± 3.8</td>
<td>0.22 ± 0.1</td>
<td>9.1 ± 4</td>
<td></td>
</tr>
<tr>
<td>E353Q (T17)</td>
<td>6.6 ± 2</td>
<td>342 ± 32.5</td>
<td>0.02 ± 0.02</td>
<td>0.019 ± 0.02</td>
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<tr>
<td>E353Q/F&lt;sub&gt;461&lt;/sub&gt;I (T7-15)</td>
<td>2.5 ± 0.4</td>
<td>42.1 ± 38.4</td>
<td>0.12 ± 0.03</td>
<td>0.12 ± 0.03</td>
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<td>E353Q/F&lt;sub&gt;461&lt;/sub&gt;I (T7-16)</td>
<td>1.2 ± 0.5</td>
<td>30.2 ± 6.7</td>
<td>0.03 ± 0.004</td>
<td>0.04 ± 0.03</td>
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<tr>
<td>E353Q (T17-2)</td>
<td>1.5 ± 1.2</td>
<td>21 ± 8.9</td>
<td>0.086 ± 1.08</td>
<td>0.09 ± 0.18</td>
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<tr>
<td>F461I</td>
<td>23 ± 0.5</td>
<td>&gt;10,000</td>
<td>0.39 ± 0.2</td>
<td>0.44 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>A569T</td>
<td>2 ± 2</td>
<td>&gt;10,000</td>
<td>0.39 ± 0.2</td>
<td>0.44 ± 0.06</td>
<td></td>
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<tr>
<td>G390D</td>
<td>25 ± 1.2</td>
<td>&gt;5000</td>
<td>0.38 ± 0.2</td>
<td>0.44 ± 0.06</td>
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<tr>
<td>L536H</td>
<td>0.84 ± 0.3</td>
<td>ND</td>
<td>0.39 ± 0.2</td>
<td>0.44 ± 0.06</td>
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<tr>
<td>G442V</td>
<td>0.65 ± 0.45</td>
<td>&gt;10,000</td>
<td>0.39 ± 0.2</td>
<td>0.44 ± 0.06</td>
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</tr>
<tr>
<td>E353Q/G390D/L536H</td>
<td>1 ± 0.9</td>
<td>ND</td>
<td>0.39 ± 0.2</td>
<td>0.44 ± 0.06</td>
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<tr>
<td>E353Q/G390D/G442V</td>
<td>0.35 ± 0.3</td>
<td>14.5 ± 10</td>
<td>0.39 ± 0.2</td>
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<tr>
<td>E353Q/G390D/A569T</td>
<td>1 ± 0.1</td>
<td>24.6 ± 15.8</td>
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<td>G442V/F&lt;sub&gt;461&lt;/sub&gt;I</td>
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<td>&gt;10,000</td>
<td>0.39 ± 0.2</td>
<td>0.44 ± 0.06</td>
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<sup>a</sup>K<sub>E2</sub> and EC<sub>50</sub> values represent the average of multiple independent determinations (n = 2–5), and the error bounds represent the range (n = 2) or S.E. (n > 2).

<sup>b</sup>RBA values were for testosterone determined with 2 nM [3H]<sub>E2</sub> for wild type and all mutants except for T7 and T17 for which 10 nM [3H]<sub>E2</sub> was used. Values represent the average of multiple independent determinations (n = 2–3).

<sup>c</sup>Testosterone binding affinity was calculated with K<sub>T</sub> = (K<sub>E2</sub>/RBA) × 100.

<sup>d</sup>ND, not determined. An EC<sub>50</sub> value could not be determined either because the plateau of dose response was not reached or because the basal level response was very high.

* High level of basal (ligand-independent) growth.

<sup>f</sup>Medium level of basal growth.
**hERα Variants with Androgen-like Specificity**

**E353Q**—Mutation E353Q was found in all evolved hERα variants. The single mutant E353Q hERα (T17) resulted in more than 29-fold higher sensitivity to testosterone and more than 11-fold lower sensitivity to E2 in yeast transactivation assay. This mutation had been identified as playing an important role in the discrimination between estrogens and androgens by site-directed mutagenesis studies (9). It was believed that the glutamate of ER (Glu-353), a strong hydrogen bond acceptor, would pair well with the A-ring phenolic group of estrogens (a strong hydrogen bond donor), whereas the corresponding glutamine of AR, a good hydrogen bond donor, would pair well with the good hydrogen bond acceptor 3-keto group of androgens (9).

**F461I**—The single mutant F461I hERα showed a 4-fold reduced sensitivity to E2 and a similar sensitivity to testosterone compared with the wild type receptor, while the double mutant E353Q/F461I hERα (T7) had dose responses to both ligands similar to the single mutant E353Q hERα (T17). Moreover, hormone binding studies indicated that T7 had an affinity for both E2 and testosterone similar to T17. Thus, F461I seemed to have no effect on the binding of testosterone to the receptor.

**G390D**—The single mutant G390D hERα had higher sensitivity to testosterone and a 4-fold lower sensitivity to E2 than the wild type hERα. In comparison, the single mutant E353Q hERα resulted in a ~29-fold higher sensitivity to testosterone and ~11-fold lower sensitivity to E2. However, the double mutant E353Q/G390D hERα showed a ~2-fold decreased sensitivity to E2 compared with the wild type hERα but a more than 476-fold higher sensitivity to testosterone than the wild type receptor. These results were in good agreement with the ligand binding affinity studies. E353Q/G390D hERα had a slightly decreased affinity to E2 (1.5-fold) relative to the wild type receptor, while E353Q hERα had a 14-fold lower affinity than the wild type receptor. Moreover, E353Q/G390D hERα had a ~16-fold higher affinity to testosterone than did E353Q hERα and a ~5,680-fold higher than did the wild type receptor. Thus, the positive effect of mutations E353Q and G390D on testosterone was synergistic, whereas when combined, their discordant effects on E2 were canceled out.

Further molecular modeling studies shed some light on the function of this G390D mutation (Fig. 5A). In the wild type ERα, the A-ring phenolic group of E2 is hydrogen bonded with both Glu-353 and Arg-394. Due to the close proximity of the side chains of Glu-353 and Arg-394 (<4 Å), the net negative charge of Glu-353 is neutralized by the positive charge of Arg-394. The resulting charge neutral ligand binding pocket binds charge neutral E2 with more favorable energetics. In T7 and T17, the substitution of glutamate by glutamine at position 353 abolishes the hydrogen bond between the wild type ERα and E2 but establishes a new hydrogen bond with testosterone, resulting in a higher affinity to testosterone and lower affinity to E2. However, since glutamine is uncharged, the positively charged Arg-394 confers on the ligand binding pocket a net positive charge, which may destabilize the receptor-ligand interactions. The substitution of glycine by aspartic acid at the neighboring residue 390 in T7-16 and T17-2 provides a negative charge, which may neutralize the positive charge on Arg-394. The combination of mutation G390D and E353Q thus further increases the affinity toward testosterone relative to that of the single mutant E353Q hERα and also increases the transactivation activity of the receptor in yeast. In addition, this charge neutral ligand binding pocket may also facilitate the binding of charge neutral E2, resulting in increased binding affinity of this mutant for E2.

**G442V**—Compared with the wild type receptor, the single mutant G442V showed a similar sensitivity to E2 and a slightly increased sensitivity to testosterone but with a moderately elevated basal level response. The double mutant G442V/F461I hERα showed the same sensitivities to both E2 and testosterone as the single mutant G442V hERα. The $K_P$ of T7-15 (E353Q/F461I/G442V) is less than half that of T7 (E353Q/F461I), while the $K_P$ of T7-15 is about one-fourth that of T7 (Table I), indicating that T7-15 can bind E2 and testosterone much more strongly than T7. In the HEC-1 cell transactivation assays, T7-15 showed a ~150-fold increased sensitivity to testosterone and a ~4-fold decreased sensitivity to E2 relative to the wild type hERα. In comparison, in yeast cell transactivation assays, T7-15 had an increased sensitivity toward both E2 and testosterone compared with T7. The double mutant E353Q/G390D was able to bind testosterone much more strongly than the wild type receptor and was also able to bind E2 with a slightly decreased $K_P$.

**Fig. 3.** Transactivation profiles for the wild type (wt) hERα and one of the second round hERα variants (T7-15) in response to E2, T, DHT, and Pg in yeast YRG2 cells (A) and HEC-1 cells (B). The values represent the mean ± S.D. or range of two or more experiments.

**Fig. 4.** Lineage and DNA sequencing results of the evolved hERα variants. WT, wild type.
G442V hERαs constructed by site-directed mutagenesis showed the same activity as T7-15 (data not shown), while the double mutant G442VF461I hERα and the single mutant G442V hERα displayed elevated basal level responses and similar dose responses relative to the wild type hERα. Thus, the contribution of mutation G442V to the increased transcription potency toward testosterone is dependent on E353Q.

Mutation G442V itself is far away from the ligand binding pocket (17.1 Å away from the A-ring phenolic group of E2 as determined from the x-ray crystal structure of hERα, Protein Data Bank code 1GWR). As shown in Figs. 1A and 5B, this mutation is located at the N terminus of helix 8 and its adjacent residue on helix 8 with its side chain pointing toward the same direction is Phe-445. These two residues form a strong van der Waals interaction between their side chains since the benzene ring of Phe-445 is only 3.5 Å above the indole ring of Trp-393 of helix 5. Furthermore, Trp-393 is located next to Arg-394, a residue that plays an important role in ligand-receptor interaction. Since the substitution of a glycine by a valine at position 442 will likely introduce residue clashes between the bulkier valine and Trp-393 (as shown in the rotamer search using MOE), it may affect the van der Waals interaction between Phe-445 and Trp-393, changing the position of Arg-394 and further altering the ligand binding affinity. Interestingly, Eng et al. (22) found that mutation G442E increased the transcription potency to E2 and 2-methoxyestrone compared with the wild type hERα in yeast. We are currently using molecular dynamic simulations to gain further insight into the role of this mutation.

L536H—The single mutant L536H hERα showed a significantly elevated basal level response to both E2 and testosterone while showing a similar sensitivity to testosterone and a slightly reduced sensitivity to E2 in yeast. The double mutant E353Q/L536H hERα (T17-4) showed a 52-fold increased transcription potency toward testosterone in HEC-1 cells (versus >640-fold in yeast) and a ~10-fold decreased potency toward E2 in HEC-1 cells (versus 2-fold in yeast) compared with the wild type hERα. The double mutant E353Q/L536H hERα had a negligible basal level response, whereas the triple mutant E353Q/G390D/L536H hERα still had an elevated basal level response.

The substitution of Leu-536 by a proline was found to result in an elevated ligand-independent response (22). Other types of substitutions, such as L536E containing a negatively charged side chain, L536K containing a positively charged side chain, L536N containing a polar side chain, and L536A or L536G containing a smaller side chain gave the same results (23). Residue Leu-536 is located at the N-terminal end of helix 12, which contains the AF-2 activation domain of hERα. It was believed that Leu-536 is critical in coupling the binding of ligand to the modulation of the conformation and activity of hERα and that a large hydrophobic residue at position 536 is necessary for ligand-dependent transactivation (23). Strikingly, our finding suggests that in the presence of mutation E353Q, L536H will increase the transcription potency of the receptor to testosterone while maintaining the receptor in an inactive state in the absence of ligand. Thus, L536H is a context-dependent mutation: only the unique combination of E353Q and L536H increased the testosterone dose response while maintaining a negligible basal level response.

A569T—The single mutant A569T hERα also showed a similar sensitivity to testosterone and a slightly decreased sensitivity to E2 relative to the wild type receptor in yeast. The triple mutant E353Q/F461I/A569T hERα (T7-18) showed a ~10-fold increased testosterone sensitivity and a ~3–4-fold increased E2 sensitivity relative to the double mutant E353Q/F461I hERα (T7) in yeast. This result suggests that A569T plays an important role in ER ligand binding.

Additivity of the Functional Mutations

Since recombining the second round hERα variants using DNA shuffling failed to yield a variant with further improved sensitivity and binding affinity to testosterone, we suspected that some of the beneficial mutations might not be additive or cumulative. Thus, we used site-directed mutagenesis to construct a few mutants containing different combinations of these functional mutations. As mentioned above, the introduction of functional mutations into the second round variant E353Q/G390D hERα (T17-2) had little effect on the transcription potency of the receptor since the triple mutants including E353Q/G390D/G442V hERα and E353Q/G390D/A569T hERα and the double mutant E353Q/G390D hERα had a similar transcription potency for both ligands. Moreover, the triple mutant E353Q/G390D/L536H hERα had a transcription potency for both ligands that was similar to that of the double mutant E353Q/L536H hERα. Thus, even though the three functional mutations (G442V, L536H, and A569T) are not in close contact with E353Q or G390D, their effects on ligand specificity are not independent and additive.
Comparison between the Evolved hERα Variants and hAR

Comparison of the x-ray crystal structures of the rat AR (Protein Data Bank code 1I37) and hERα revealed a very high degree of structural homology (Fig. 6A). There are a total of 20 amino acid residues in hER and hAR that interact with the bound ligand (either E2 or testosterone) (Figs. 1A and 6B). Of them, only six are identical between hER and hAR, even though 18 are located at the same positions in the three-dimensional structures. Most of the residues are hydrophobic and interact with the ligand scaffold primarily through van der Waals interactions, whereas a few residues are polar and form hydrogen bonds to the polar atoms in the ligand. For example, in hERα, polar residues Glu-353, Arg-394, and His-524 form hydrogen bonds with the hydroxyl groups at both ends of E2, while in hAR, residues Gln-711, Arg-752, and Thr-877 form corresponding hydrogen bonds with testosterone. The remaining 17 residues in both receptors, except for hERα Thr-347 and hAR Asn-705, are hydrophobic and interact with the ligand scaffold through van der Waals interactions. It should be noted that the number of residues that interact with the ligand may vary depending on the chemical structure of a specific ligand. For example, more residues in hERα interact with raloxifene than with E2 (24).

As mentioned above, mutation E353Q alters the hydrogen bond interaction of hERα with estrogens and androgens, and of the five discovered beneficial mutations, E353Q is the only residue that directly interacts with the ligand in hERα. The other four mutations are not located in the ligand binding pocket in either receptor, and none of them except for F461I has an equivalent residue at the corresponding position in hAR. It should be noted that the corresponding residue for mutation L536H in rat AR is Phe-891, which directly interacts with testosterone through van der Waals interaction.

Taken together, these results suggest that the significantly enhanced androgen specificity and affinity in the evolved hERα variants may be obtained through subtle modulation of the van der Waals interactions and probably hydrogen bonds between the receptor and ligand by many residues far away from the ligand binding pocket. Very recently, such long range interactions were also demonstrated to be important in determining the ligand selectivity of human estrogen receptor subtypes (25). Due to the subtlety and complexity of these long range interactions, it is not surprising that some independently obtained functional mutations are not additive or cumulative in their effect on ligand specificity and affinity.

Implications in Molecular Evolution of Nuclear Receptors

There are six evolutionarily related nuclear receptors for steroid hormones: two for estrogens (ERα and ERβ) and one each for androgens (AR), progestins (progesterone receptor), glucocorticoids (glucocorticoid receptor), and mineralocorticoids (mineralocorticoid receptor). Molecular phylogenetic analysis suggests that all these steroid receptors have evolved from an ancestral estrogen receptor through a series of gene duplication and divergence events (26, 27). Furthermore, a ligand exploitation model was proposed as the evolutionary mechanism for steroid receptors in which the terminal ligand (estrogens) in the steroid biosynthetic pathway is the first for which a receptor (an estrogen receptor) evolves; selection for this hormone also selects for the synthesis of intermediates (e.g. progesterone and testosterone) despite the absence of receptors, and duplicated receptors then evolve affinity for these precursor substances (27). This model was corroborated by most recent work on the isolation, reconstruction, and characterization of the lost ancestral steroid receptor (28).

Consistent with this model, our directed evolution studies on hERα indicate that significantly enhanced androgen affinity could be readily created in the hERα LBD by two rounds of random point mutagenesis followed by selection for increased affinity to testosterone. Strikingly, only two mutations are needed to achieve near nanomolar affinity for testosterone, and more strikingly, most functional mutations are not in contact with the ligand. Nevertheless, the only ligand-contacting mutation E353Q does play a key role in conferring androgen affinity. Multiple sequence alignment analyses of ARs and ERs showed that a residue corresponding to Gln-353 is found in all naturally occurring androgen receptors (35 AR sequences from 28 different species, Supplemental Fig. 8A), whereas this same position harbors a glutamate residue in all naturally occurring estrogen receptors (34 ERα sequences from 32 different species and 42 ERβ sequences from 32 different species; Supplemental Fig. 8, B and C). This suggests that evolution of a unique glutamine at this position in the ancestral AR sequence (evolved from an ancient ancestral ER sequence (28)) might have been a key event in the emergence of a receptor that binds...
steroid receptor toward new ligand affinities and that there
evolution and further suggest that both positive and negative
evidence for the ligand exploitation model of steroid receptor
slightly decreased affinity for E2 and thus became promiscuous
significantly enhanced binding affinity for testosterone and only
dominant role in discriminating between E2 and testosterone.
(18 of 20). The only ligand-contacting mutation E353Q plays a
and are located at the same positions in the crystal structures
contacting residues in both receptors are hydrophobic (16 of 20)
assays, and Jerome Baudry for help with the molecular modeling stud-
mammalian cell assays, Kathryn Carlson for help with the hormone binding
or ERs, the laboratory-evolved hER
33864
testosterone. Furthermore, unlike the naturally occurring ARs
ligand class promiscuity might play an important role in the
creation of new receptors by providing a head start toward
being captured by adaptive evolution with further mutations
providing the required specificity toward the newly adopted
ligand. Of note, a similar evolutionary mechanism was pro-
posed for the creation of new enzyme activities via divergent
evolution (29).

Conclusions

The outcome of the directed evolution studies described
above is five hERα variants with up to 7,600-fold improvement
in the binding affinity for testosterone (38–95 nM) and a
slightly reduced affinity for E2 (0.44–0.9 nM). Such dramatic
affinity alteration to testosterone required only two or three
mutations, and only one of the five beneficial mutations iden-
tified from directed evolution was in direct contact with the
ligand. Several of the mutations were synergistic or context-
dependent. Structural comparison between these evolved
hERα variants and hAR indicated that most of the ligand-
contacting residues in both receptors are hydrophobic (16 of 20)
and are located at the same positions in the crystal structures
(18 of 20). The only ligand-contacting mutation E353Q plays a
dominant role in discriminating between E2 and testosterone.
However, subtle changes in the interactions between the recep-
tor and ligand, brought by the mutations far away from the
binding pocket, can also significantly affect the ligand affinity.
Such in vitro evolution studies provide strong experimental
evidence for the ligand exploitation model of steroid receptor
evolution and further suggest that both positive and negative
selection may operate simultaneously in the evolution of a
steroid receptor toward new ligand affinities and that there
may be several evolutionary pathways toward the same end.

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REFERENCES

529–536
2. Mangelsdorfer, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono,
835–839
1325–1341
6. Nilsson, S., Makela, S., Treuter, E., Tuuga, M., Thomsen, J., Andersson, G.,
Physiol. Rev. 81, 1535–1565
7. Mophaul, M. J., Marcelli, M., Tilley, W. D., Griffin, J. E., and Wilson, J. D.
macol. Res. 32, 217–221
Chem. 273, 693–699
24089–24096
13. Ding, X. F., Anderson, C. M., Ma, H., Heng, H., Uht, R. M., Kushner, P. J., and
Industrial Microbiology and Biotechnology (Demain, A. L., and Davies, J. E., eds) 2nd Ed., pp. 597–604, American Society for Microbiology Press,
Washington, D. C.
19. Muthyala, R. S., Sheng, S. B., Carlsson, K. E., Katzenellenbogen, B. S., and
istry 12, 4085–4092
Cell. Biol. 17, 4644–4653
23. Zhao, C., Koide, S., Abrams, J., Deighton-Collins, S., Martinez, A., Schwartz,
24. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom,
Nature 389, 753–758
317–327