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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 nonadditive. 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)[1] and androgen receptor (AR) belong to the steroid hormone nuclear receptor superfamily that regulates hormone-responsive genes in a ligand-inducible manner (1). Like other members of this nuclear receptor superfamily, the ER and AR have three modular structural domains: an amino-terminal ligand-independent transactivation domain, a central DNA binding domain, and a carboxy-terminal ligand binding domain (LBD). The LBD of either ER or AR interacts specifically with its physiological ligand and contains a dimerization function and a ligand-dependent activation function AF-2 (2–5). Interestingly, despite their low sequence homology (<20%), the ER LBD and the AR LBD share a similar structural motif consisting of 12 α-helices arranged in an antiparallel sandwich motif (Figs. 1*A* and 6*A*).

Both the ER and the AR are of particular interest because of their important roles in the growth, differentiation, metabolism, reproduction, and morphogenesis of higher organisms and humans and their association with several human diseases. Estrogens such as 17β-estradiol (E_2) (Fig. 1*B*), acting through the ERα or ERβ, regulate the differentiation and maintenance of neural, skeletal, reproductive, and cardiovascular tissues. The regulation of ERα activity plays a critical role in osteoporosis, cardiovascular disease, and breast cancer (3, 6). Androgens and their receptors exert crucial actions in male physiology and pathology. The binding of male sex steroids 5α-dihydrotestosterone (DHT) and testosterone (T) (Fig. 1*B*) to the AR initiates male sexual differentiation and development (7). Mutations in the human AR LBD have been linked to several diseases such as prostate cancer and the androgen insensitivity syndrome (3). Thus, considerable effort has been directed at elucidating the molecular basis of ligand specificity of the ER and AR LBDs.

The ligand specificity and sensitivity of the ER or AR toward its physiological ligand are extremely high. Both the AR and the ER bind their physiological ligands with subnanomolar affinity, and a small difference in the ligand structure can have a dramatic effect on ligand binding affinity. For example, although the chemical structures of testosterone and E_2 differ only slightly in the A-ring region (Fig. 1*B*), the affinity of human ERα for testosterone is >10,000-fold weaker than that for E_2 (this work), whereas the affinity of human AR for E_2 is 44-fold weaker than that for testosterone (8). Comparison of the crystallographic structures of the human ERα LBD (hERα LBD) complexed with E_2 and the human AR LBD (hAR LBD) complexed with testosterone indicates that the majority of the residues (14 of 20) interacting with the ligand are different between the ER and the AR (Fig. 1*A*). Thus, we have been

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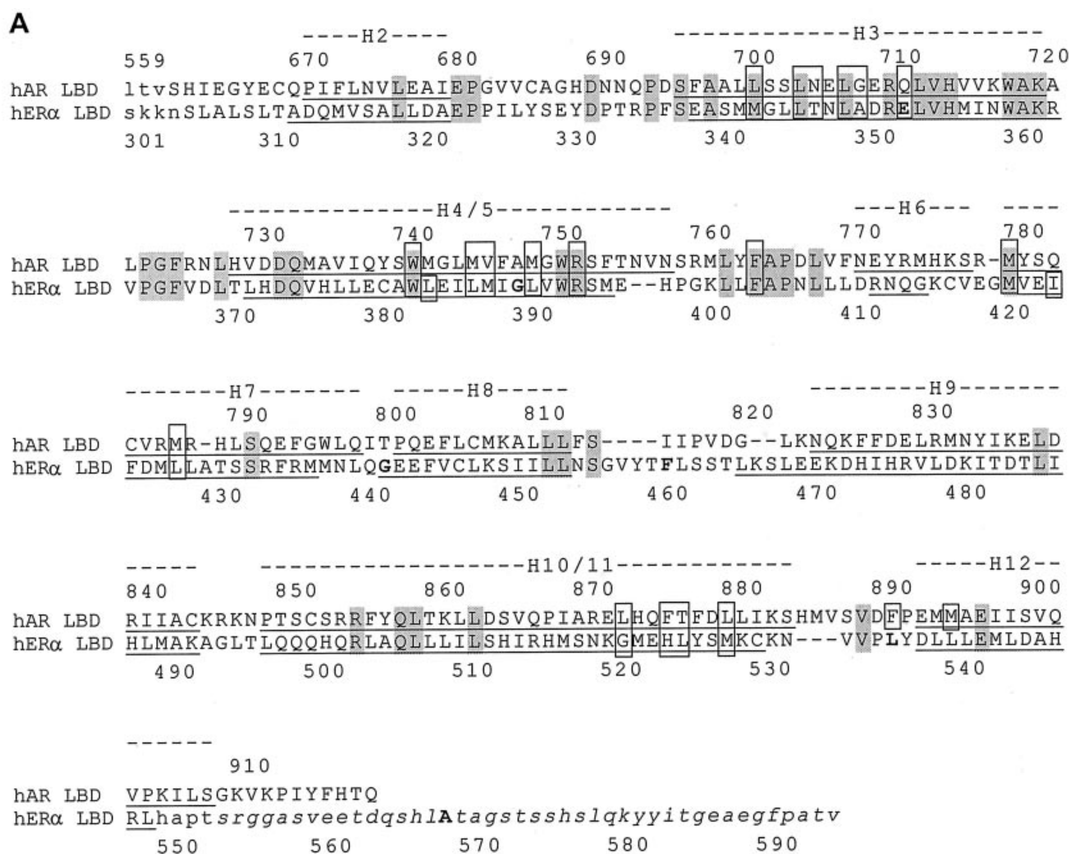
[1] The abbreviations used are: ER, estrogen receptor; AR, androgen receptor; DHT, 5α-dihydrotestosterone; ERE, estrogen response element; EC_{50}, half-maximal effect concentration; E_2, 17β-estradiol; HEC-1, human endometrial cancer; LBD, ligand binding domain; MOE, molecular operating environment; RBA, relative binding affinity; SRC-1, steroid receptor coactivator 1; T, testosterone; Pg, Progesterone; h, human.



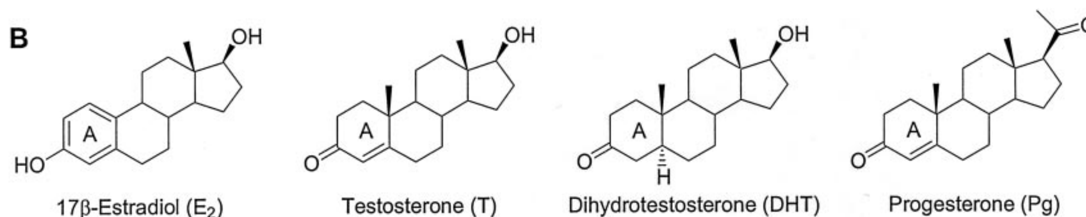


FIG. 1. *A*, sequence alignment between hERα LBD and hAR LBD. Residues not included in the crystal structures are signified by *lowercase letters*. Residues shown in *italicized lowercase letters* are hERα F domain residues. The *residue number* applies to the residue directly *above* or *below* the *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 E_2, T, DHT, and Pg. *H2–H12*, helices 2–12.

intrigued by the structural features of hERα and hAR that underlie their ability to discriminate E_2 from testosterone. Prior site-directed mutagenesis studies have indicated that the hERα 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 hERα variants with up to 7,600-fold improvement in the binding affinity toward testosterone. Strikingly, these evolved hERα variants exhibited only slightly reduced binding affinity (1.5–3-fold) to 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 17β-estradiol, testosterone, dihydrotestosterone, and isopropyl-β-D-thiogalactopyranoside were from Sigma. All restriction enzymes and DNA-modifying enzymes were obtained from New England BioLabs (Beverly, MA). Yeast strain YRG-2 (Mat a *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-538* LYS2::UAS_{GAL1}-$TATA_{GAL1}$-HIS3 URA3::$UAS_{GAL4\ 17mers(x3)}$-$TATA_{CYC1}$-lacZ) were from Stratagene (La Jolla, CA). *Taq* DNA polymerase was from Promega Corp. [^{3}H]E_2 (50 Ci/mmol) was obtained from Amersham Biosciences. QIAprep spin plasmid miniprep kit, QIAEX II gel purification kit, and QIAquick PCR purification kit were purchased from Qiagen (Valencia, CA). Various

oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA).

Plasmid Construction—pCMV5-ERα plasmid containing the wild type full-length hERα cDNA (12) was first digested with EagI 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 hERα containing hERα amino acids 312–595. pGAD424 SRC-1 containing the full-length SRC-1 was constructed as described elsewhere (13). Mammalian cell reporter plasmid $(ERE)_2$-pS2-Luc encoding a luciferase gene downstream of 2ERE was described in Ref. 14, and the internal control plasmid pCMVβ was from Clontech.

The genes encoding the evolved hERα LBD variants were subcloned into the pCMV5-ERα plasmids to reconstitute the full-length hERα genes. Briefly the LBD genes were PCR-amplified with primers ERαLBDCam5F (5′-ggc cga cca gat ggt cag tg-3′) and ERα3_ClaI (5′-gga tcg att cag act gtg gca g-3′, the ClaI restriction site is underlined), and the PCR products were purified using a QIAquick PCR purification kit. The pCMV5-ERα plasmid was digested with HindIII, and the 1-kb fragment was purified from a 1% agarose gel. The two purified DNA fragments share an overlap region of approximately 100 bp. Overlap extension PCR was performed to obtain full-length mutant hERα genes (10 cycles of 94 °C for 1 min and 72 °C for 4 min) followed by PCR amplification using primer ERα_pCMV5_5KpnI (5′-ccg gta ccc cat gac cat gac-3′, the KpnI restriction site is underlined) and ERα3_ClaI. The PCR products were then purified, digested with KpnI and ClaI, and ligated into the KpnI-ClaI-digested pCMV5-ERα vector.

The LBDs of the wild type hERα and the evolved hERα variants were also subcloned into pET15b (Novagen) for protein expression in *Escherichia coli*. Briefly, for each plasmid construct, the LBD gene was amplified from pBD-GAL4 hERα (wild type or mutants) with primers ERα_NdeI_N (5′-act gta tcg ccg cat atg gcc gac cag atg-3′, the NdeI site is underlined) and ERα_BamHI_C (5′-taa gcg gga tcc tca gac tgt ggc agg gaa-3′, the BamHI site is underlined), purified, digested with NdeI and BamHI, and ligated into the NdeI-BamHI-digested pET15b vector.

Library Creation and Screening—Error-prone PCR was used to generate a library of mutagenized LBD fragments consisting of hERα amino acids 312–595 using pBD-GAL4 hERα as a template and ERαLBDCam5F and ERαLBDCam3R (5′-tca gac tgt ggc agg gaa acc-3′) as primers according to the protocol described elsewhere (15). The resulting mutagenized LBD fragments were then co-transformed with the pBD-GAL4 hERα vector that was digested with BglII and BsaI to form a 94-bp gap into yeast YRG2 cells pretransformed with the pGAD424 SRC-1 plasmid. Yeast transformation was carried out using the lithium acetate/single-stranded carrier DNA/polyethylene 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 mutagenized LBD fragments were co-transformed with the BglII-BsaI-digested pBD-GAL4 hERα vector into *Saccharomyces cerevisiae* YRG2 cells harboring pGAD424 SRC-1. The transformed cells were plated on an agar plate containing minimum medium lacking tryptophan, leucine, and histidine and supplemented with an appropriate testosterone concentration. The testosterone concentration was chosen such that yeast cells bearing the parental hERα LBD in each round of directed evolution cannot form colonies, whereas yeast cells bearing a variant with 5–10-fold improvement in ligand binding affinity may form colonies. Colonies that formed on the selection plates after incubation at 30 °C for 2 days were picked and streaked onto two agar plates containing the same minimum medium as mentioned above, one with and one 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 EC_{50} values. In the 96-well plate assay, yeast YRG2 cells were grown to log phase (A_{600} = 2–5) in synthetic complete minimal medium lacking leucine and tryptophan at 30 °C overnight (12–16 h) with shaking. The resulting cell culture was diluted to $A_{600} = 0.002$ in liquid minimal medium lacking leucine, tryptophan, and histidine. Each well in the 96-well plate contained 200 μl of diluted yeast cells and 0.2 μl of the appropriate ligand at a specific concentration dissolved in pure dimethylformamide or EtOH. 96-well plates were incubated at 30 °C for 20–24 h, and the cell density at 600 nm was measured using a SpectraMax 340 PC plate reader (Amersham Biosciences).

Site-directed Mutagenesis—The single, double, and triple mutants were created using overlap extension PCR and yeast *in vivo* recombination. Briefly, for each mutation, a pair of mutagenic primers with each containing the desired mutation was prepared and used in combination with two oligonucleotide primers flanking the LBD gene. Each of these two flanking primers contained 20 bp homologous to the linearized pBD-GAL4-Cam vector at its 5′-end. Two separate PCRs were carried out, each containing one flanking primer and one mutagenic primer. The two PCR products were purified from a 1% agarose gel after DNA electrophoresis, mixed together in an equal molar ratio, and assembled by a primerless PCR. The assembled products were amplified with the two flanking primers. The resulting PCR product was purified from the gel and cotransformed into yeast YRG-2 cells with the EcoRI-SalI-digested pBD-GAL4-Cam vector to obtain circular plasmids. Plasmids were rescued from yeast using the Zymoprep kit (Zymo Research Corp.) and transformed into *E. coli* DH5α to obtain plasmid DNA with high purity. For single mutants, the wild type LBD gene was used as the template for the overlap extension PCR, while for double and triple mutants, the corresponding single and double mutant LBD genes were used as the template, respectively. DNA sequencing was carried out to confirm the presence of the introduced specific mutations and the absence of PCR-associated random mutations. The sequences of the primers will be provided upon request.

DNA Shuffling and DNA Sequencing—DNA shuffling of the second round hERα variants was carried out as described elsewhere (17). DNA sequencing of the evolved hERα 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 pET15b plasmid containing the wild type or mutant hERα LBD fragments encoding residues 312–595 were grown at 37 °C until A_{600} = 0.6. Protein expression was then induced with 0.5 mM isopropyl-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 ml/g of wet cells, and lysed by a French press according to the standard protocol suggested by the manufacturer. The cell debris were separated from the supernatants by centrifugation for 30 min at 10,000 × *g*. The resulting supernatants 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 purified proteins were used in the assays. Relative binding affinity (RBA) measurements were determined as previously reported (20) with slight modifications. The cell extracts containing wild type or mutant hERα were diluted to 1 nM in buffer B and incubated with buffer alone or with several concentrations of unlabeled competitor together with 2 or 10 nM $[^3H]E_2$ at 0 °C for 16–24 h. At the end of the incubation, the solutions were processed the same way as in the equilibrium ligand binding assay. The unlabeled competitors were diluted to a 1:1 ratio of dimethylformamide:buffer B to ensure solubility, and the final concentration of dimethylformamide in the solution was 7%.

Molecular Modeling—The hERα LBD crystal structure (Protein Data Bank code 1GWR) was imported into MOE (Molecular Operating Environment, Chemical Computing Group Inc., Montreal, Quebec, Canada). Force field MMFF94s was used for all simulations. After fixing all missing residues and atoms manually, hydrogen atoms were added. Two steps of energy minimization were performed, first with all the α-carbons fixed and then without constraints. Residue Glu-353 was mutated to Gln using the MOE Rotamer Explorer function, and the rotamer that adopts a similar conformation as the original Glu was selected. Testosterone was built using the MOE Builder function and energy-minimized. This testosterone structure was superimposable with the DHT structure found in the rat AR LBD crystal structure complexed with DHT (Protein Data Bank code 1I37). Testosterone was manually placed into the ligand binding pocket of the mutated hERα (E353Q) followed by running the docking program (initial temperature, 5000 K; iteration limit, 8000 cycles; 10 cycles per run; 25 runs). The lowest energy docked conformation was further energy-minimized with atoms more than 10 Å away from the ligand being fixed. A short molecular dynamic simulation was then carried out to release all the energy constraints caused by ligand docking. During the molecular dynamic simulation, the energy-minimized receptor-ligand complex was heated to 310 K at 100 K/ps and equilibrated at 310 K for 20 ps with time steps of 1 fs and atoms more than 10Å away from the ligand being fixed. For the structural comparison between AR and ER, the structures of rat AR (Protein Data Bank code 1I37)[2] and hERα (Protein Data Bank 1GWR) were imported into MOE and superimposed based on sequence homology.

[2] Since the protein sequences for rat and human AR LBD are identical, the affinity value and crystal structure of the rat AR were used for the human AR in this report.

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* YRG-2, 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 biosynthesis 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 A_{600} (cell density) was measured. First, it was shown that cells bearing plasmids pBD-GAL4 hERα and pGAD424 SRC-1 responded to E_2 at a subnanomolar concentration, while cells bearing plasmid pBD-GAL4 ERα alone had no response to E_2 up to a micromolar concentration, indicating that the cell growth assay is tripartite (Fig. 2*A*). 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 (E_2 (100) > 17α-estradiol (58) > 2-OH-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. 2*B*). These results indicate this selection/screening method is very sensitive and specific. It should be noted that the half-maximal effect concentration (EC_{50}, the ligand concentration that causes a half-maximal response) of the wild type hERα LBD for E_2 was estimated to be ~0.6 nM, which is in good agreement with the reported value of the full-length hERα for E_2 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 EC_{50} values. Moreover, to ensure that the improved EC_{50} 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 EC_{50} value could be obtained.

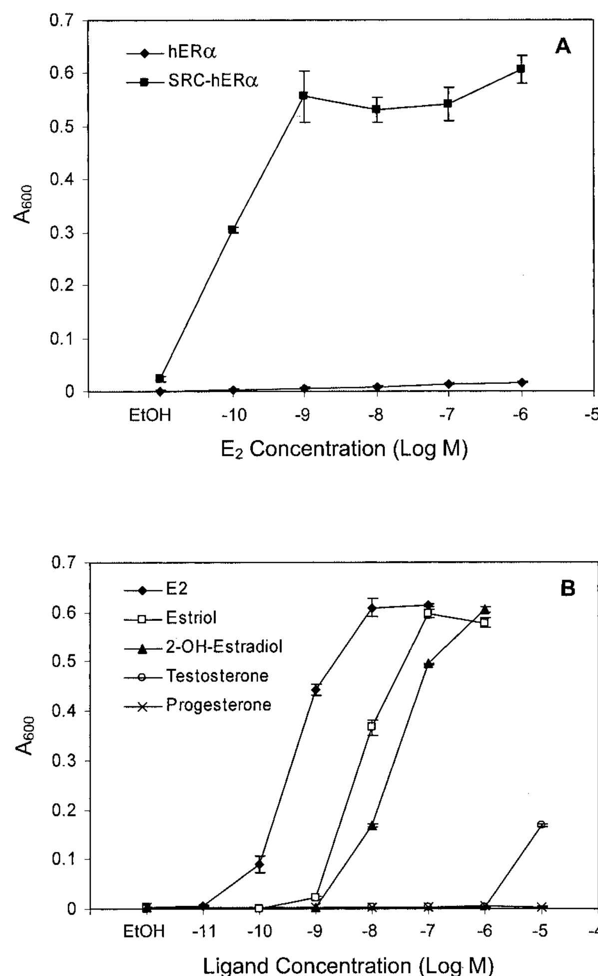


FIG. 2. **Experiments designed to validate the yeast two-hybrid-based high throughput screening method.** *A*, comparison of dose responses of YRG2 cells bearing pBD-GAL4 hERα and pGAD424 SRC-1 and YRG2 cells bearing plasmid pBD-GAL4 hERα alone. *B*, comparison of dose responses of a panel of diverse ligands to the wild type hERα.

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-

ing from T7 were grown with 10^{-8} M testosterone, and 19 clones were selected. Three of them (T7-15, T7-16, and T7-18) were identified 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 EC_{50} 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 E_2, 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 E_2 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 mutants (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 LBD variants (Fig. 4). For the first round variants, T17 had a single mutation, E353Q, whereas T7 had two mutations, E353Q and F461I. Since both variants had a similar ligand dose response to either testosterone or E_2, F461I was thought to be functionally neutral. For the second round variants, since they all contained only one new mutation compared with their parental genes, the following mutations could be unequivocally identified as responsible for the observed higher testosterone response: G442V, G390D, A569T, and L536H. It is of note that G390D appeared in two mutants from two independent libraries. It should also be noted that DNA shuffling of these second round variants failed to produce a variant with further increased response toward testosterone.

To further investigate the effects of these mutations on 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 E_2 and a similar sensitivity to testosterone, whereas the single mutant G390D hERα showed increased sensitivity to testosterone and a slightly reduced sensitivity to E_2. The single mutant G442V hERα showed a similar sensitivity to E_2 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 E_2. The single mutant A569T hERα also showed a similar sensitivity to testosterone and a slightly decreased sensitivity to E_2. The double mutant G442V/F461I hERα showed sensitivities to both E_2 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 E_2 but showed a very high basal level response, which made the estimation of EC_{50} 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 E_2 (K_d^E) were determined by a direct hormone binding assay. The K_d^E of the wild type hERα was 0.29 ± 0.02 nM, whereas the evolved hERα variants had increased K_d values toward E_2, 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 E_2 for the wild type hERα is set at 100. The RBA of testosterone for the wild type hERα was less than 10^{-4}, 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 (K_d^T) 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 nonconservative changes. All these mutations were mapped into the crystal structure of the hERα-testosterone complex in our molecular modeling studies.

TABLE I
Summary of the responses of mutant estrogen receptor to E_2, T, DHT, and Pg in yeast and HEC-1 cells

Estrogen receptor	EC_{50}[a]								K_d^{E2} (n)[a]	RBA^T (n)[c]	K_d^{T}[c]
	Yeast				HEC-1						
	E_2	T	DHT	Pg	E_2	T	DHT	Pg			
			nM				*nM*		*nM*		*nM*
Wild type	0.6 ± 0.14	>10000	833 ± 382	>10,000	0.032 ± 0.01	1400 ± 438	378 ± 196	>10,000	0.29 ± 0.02 (5)	$<10^{-4}$ (3)	>290,000
E353Q/F461I (T7)	9.9 ± 4.1	257 ± 38	11 ± 8.3	5816 ± 2775	0.22 ± 0.1	ND[d]	9.1 ± 4	1287 ± 67	2.21 ± 0.46 (4)	0.51 ± 0.02 (2)	431
E353Q (T17)	6.6 ± 2	342 ± 32.5	12.4 ± 11	6402 ± 1447	0.19 ± 0.02	51 ± 5.4	17.7 ± 9.2	2568 ± 492	3.53 ± 1.54 (4)	0.51 ± 0.13 (2)	692
E353Q/F461I/G442V (T7-15)	2.5 ± 0.4	42.1 ± 28.4	0.81 ± 0.12	789 ± 7.2	0.12 ± 0.03	9 ± 5.2	2.2 ± 0.51	917 ± 68	0.9 ± 0.08 (2)	0.95 ± 0.19 (2)	95
E353Q/F461I/G390D (T7-16)	1.2 ± 0.5	30.2 ± 6.7	0.45 ± 0.02	292 ± 8.8	0.033 ± 0.004	4.4 ± 0.3	0.48 ± 0.08	490 ± 343	0.5 ± 0.15 (2)	0.95 ± 0.32 (3)	52
E353Q/F461I/A569T (T7-18)	3.1 ± 1.8	25.1 ± 16.4	0.64 ± 0.23	479 ± 114	0.88 ± 0.5	18.3 ± 1.8	4.5 ± 0.4	1244 ± 299	0.58 ± 0.04 (2)	1.52 ± 0.23 (3)	38
E353Q/G390D (T17-2)	1.5 ± 1.2	21 ± 8.9	0.37 ± 0.02	402 ± 148	0.086 ± 1.08	18.7 ± 19.2	2.6 ± 0.2	1020 ± 30	0.44 ± 0.06 (3)	0.88 ± 0.21 (3)	51
E353Q/L536H (T17-4)	1.3 ± 0.24	16.3 ± 3.9	0.56 ± 0.4	362 ± 162	0.39 ± 0.2	27.1 ± 15	3.6 ± 0.9	3852 ± 1270	0.69 ± 0.22 (2)	1.52 ± 0.22 (3)	45
F461I	2.3 ± 0.5	>10,000									
A569T	2 ± 2	>10,000									
G390D	2.5 ± 1.2	>5000									
L536H[e]	0.84 ± 0.3	ND									
G442V[f]	0.65 ± 0.45	>10,000									
E353Q/G390D/L536H[e]	1 ± 0.9	ND									
E353Q/G390D/G442V	0.35 ± 0.3	14.8 ± 10									
E353Q/G390D/A569T	1 ± 0.1	24.6 ± 15.8									
G442V/F461I[f]	0.2 ± 0.2	>10,000									

[a] K_d and EC_{50} values represent the average of multiple independent determinations (n = 2–5), and the error bounds represent the range (n = 2) or S.E. ($n > 2$).
[b] RBA values were for testosterone determined with 2 nM $[^3H]E_2$ for wild type and all mutants except for T7 and T17 for which 10 nM $[^3H]E_2$ was used. Values represent the average of multiple independent determinations (n = 2–3).
[c] Testosterone binding affinity was calculated with $K_d^{T} = (K_d^{E2}/RBA) \times 100$.
[d] ND, not determined. An EC_{50} value could not be determined either because the plateau of dose response was not reached or because the basal level response was very high.
[e] High level of basal (ligand-independent) growth.
[f] Medium level of basal growth.

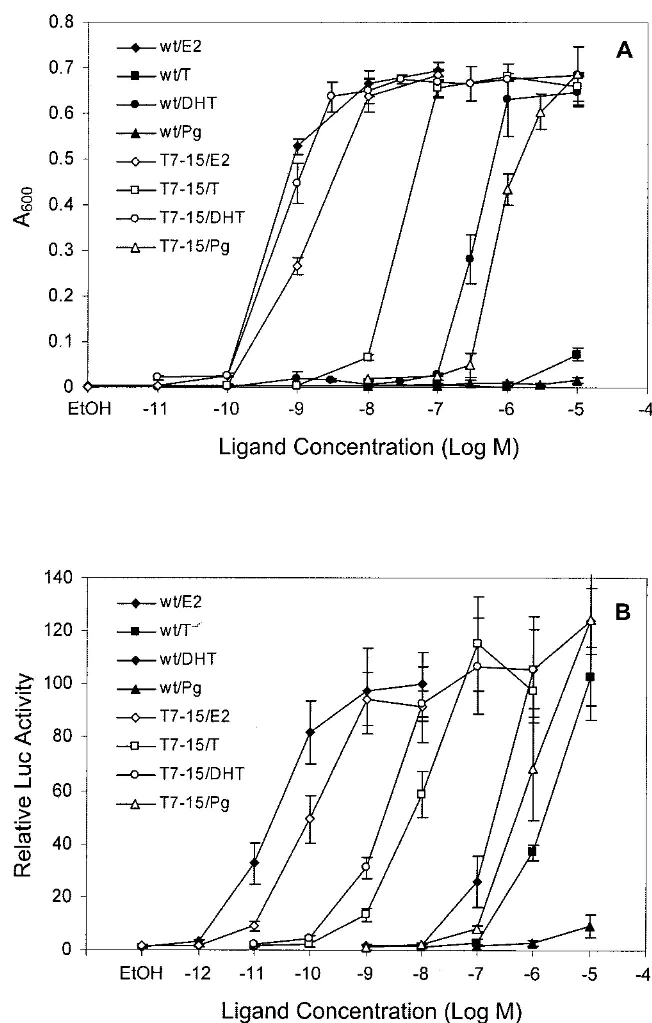


FIG. 3. **Transactivation profiles for the wild type (*wt*) hERα and one of the second round hERα variants (T7-15) in response to E_2, T, DHT, and Pg in yeast YRG2 cells (*A*) and HEC-1 cells (*B*).** The values represent the mean ±S.D. or range of two or more experiments.

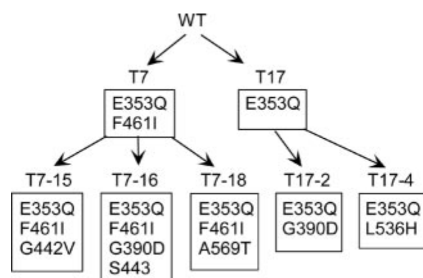


FIG. 4. **Lineage and DNA sequencing results of the evolved hERα variants.** *WT*, wild type.

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 ~11-fold lower sensitivity to E_2 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 E_2 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 E_2 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 E_2 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 E_2. However, the double mutant E353Q/G390D hERα showed a ~2-fold decreased sensitivity to E_2 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 E_2 (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 E_2 were canceled out.

Further molecular modeling studies shed some light on the function of this G390D mutation (Fig. 5*A*). In the wild type hERα, the A-ring phenolic group of E_2 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 E_2 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 hERα and E_2 but establishes a new hydrogen bond with testosterone, resulting in a higher affinity to testosterone and lower affinity to E_2. 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 E_2, resulting in increased binding affinity of this mutant for E_2.

G442V—Compared with the wild type receptor, the single mutant G442V showed a similar sensitivity to E_2 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 E_2 and testosterone as the single mutant G442V hERα. The K_d^E of T7-15 (E353Q/F461I/G442V) is less than half that of T7 (E353Q/F461I), while the K_d^T of T7-15 is about one-fourth that of T7 (Table I), indicating that T7-15 can bind E_2 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 E_2 relative to the wild type hERα. In comparison, in yeast cell transactivation assays, T7-15 had an increased sensitivity toward both E_2 and testosterone compared with T7. The double mutant E353Q/

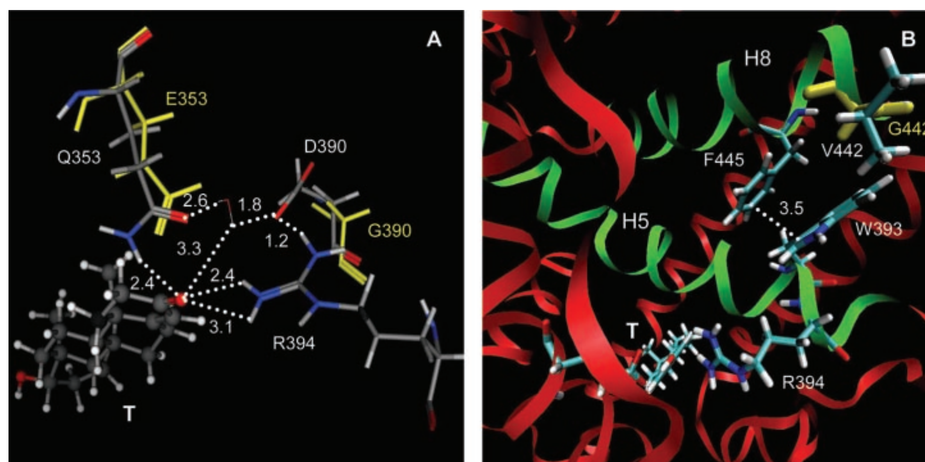


FIG. 5. **The structural model showing the G390D mutation (*A*) and the G442V mutation (*B*) and their interacting residues and the ligand.** The wild type residues are shown in *yellow*, and the mutated residues are shown in their *element color*. The structural model and the various hydrogen bonds in *A* were generated using MOE. The structural model in *B* was made using the Visual Molecular Dynamics program (30). *H5*, helix 5; *H8*, helix 8.

G442V hERα constructed by site-directed mutagenesis showed the same activity as T7-15 (data not shown), while the double mutant G442V/F461I 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 E_2 as determined from the x-ray crystal structure of hERα, Protein Data Bank code 1GWR). As shown in Figs. 1*A* and 5*B*, 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 E_2 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 E_2 and testosterone while showing a similar sensitivity to testosterone and a slightly reduced sensitivity to E_2 in yeast. The double mutant E353Q/L536H hERα (T17-4) showed a 52-fold increased transcriptional potency toward testosterone in HEC-1 cells (*versus* >640-fold in yeast) and a ~10-fold decreased potency toward E_2 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 E_2 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 E_2 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.

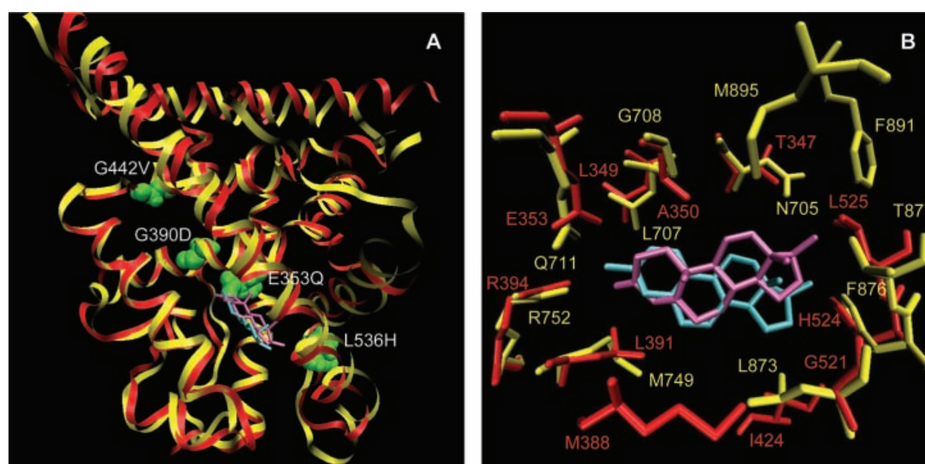


FIG. 6. *A*, ribbon diagrams of the superimposed three-dimensional structures of hAR LBD (*yellow*) complexed with testosterone (*cyan*) and hERα LBD (*red*) complexed with E_2 (*pink*). The beneficial mutations except for A569T are shown in Corey-Pauling-Koltun model (*green*). A569T is not in the LBD domain. *B*, diagrams showing the interactions between the ligand (testosterone or E_2) with the residues within the binding cavity (van der Waals cut-off distance, 4.0 Å) and the receptor (hAR LBD or hERα LBD). Residues Met-343, Leu-346, Trp-383, Phe-404, Met-421, Leu-428, and Met-528 from hERα LBD and Leu-701, Trp-741, Phe-764, Met-780, and Met-787 from hAR LBD are omitted from the graph for clarity. Both diagrams were made using Visual Molecular Dynamics (30).

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. 6*A*). There are a total of 20 amino acid residues in hER and hAR that interact with the bound ligand (either E_2 or testosterone) (Figs. 1*A* and 6*B*). 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 E_2, 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 E_2 (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

testosterone. Furthermore, unlike the naturally occurring ARs or ERs, the laboratory-evolved hERα variants exhibited significantly enhanced binding affinity for testosterone and only slightly decreased affinity for E_2 and thus became promiscuous receptors at least as far as estrogens and androgens are concerned. Since members in the steroid receptor family arose from a common ancestor via divergent evolution, such dual 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 proposed 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 E_2 (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 identified 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 E_2 and testosterone. However, subtle changes in the interactions between the receptor 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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