Estrogens, primarily 17β-estradiol (E2), may play important roles in male physiology via the androgen receptor (AR). It has already been shown that E2 modulates AR function in LNCaP prostate cancer cells and xenograft CWR22 prostate cancer tissues. Using a molecular model of E2-bound AR-ligand binding domain (LBD) and employing site-directed mutagenesis strategies, we screened several AR mutants that were mutated at E2-AR contact sites. We found a mutation at amino acid 749, AR(M749L), which confers AR hypersensitivity to E2. The reporter assays demonstrate that E2 can function, like androgen, to induce AR(M749L) transactivation. This E2-induced AR mutant transactivation is a direct effect of the AR(M749L), because the transactivation was blocked by antiandrogens. The hypersensitivity of AR(M749L) to E2 is not due to increased affinity of AR(M749L) for E2, rather it may be due to the existence of the proper conformation necessary to maintain E2 binding to the AR-LBD long enough to result in E2-induced transactivation. AR(M749L) transactivation can be further enhanced in the presence of AR coregulators, such as ARA70 and SRC-1. Therefore, amino acid 749 may represent an important site within the AR-LBD that is involved in interaction with E2 that, when mutated, allows E2 induction of AR transactivation.

Estrogens play an important role in the normal and abnormal processes of male physiology (1). Male estrogen is synthesized by aromatization of the principle androgen, testosterone, in many tissues, including brain, liver, adipose tissue, and prostate. The physiological level of circulating 17β-estradiol (E2) in the adult male is ∼0.1 nM (∼73–184 pg/ml or 12–34 pg/ml) (2), however, local aromatase activity may cause tissue levels to be higher than the serum level. Estrogens exert feedback control at the level of the hypothalamus and pituitary by decreasing luteinizing hormone-releasing hormone and luteinizing hormone production. The necessity of estrogens for male fertility was discovered in aromatase knockout (3) and estrogen receptor α (ERα) knockout (4) mice. Both strains of mice develop infertility and exhibit defects of the reproductive system. Furthermore, estrogen imprinting on male reproductive organs causes poor semen quality, cryptorchidism, and testicular and prostatic hyperplasia in male offspring with prenatal exposure to high levels of estrogen (5).

Isolation and Characterization of Androgen Receptor Mutant, AR(M749L), with Hypersensitivity to 17β Estradiol Treatment*

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EXPERIMENTAL PROCEDURES

Chemicals—E2 (Sigma), [3H]E2 (3H-labeled (2,4,6,7)-17β-estradiol, specific activity 118 Ci/mmol, PerkinElmer Life Sciences, Boston, MA), and trypsin (Promega) were purchased. ICI 176,334 (Micronised, Adm: 44008/90 C:41567, Imperial Chemical Industries, PLC Pharmaceuticals Division, Great Britain) was kindly provided by Dr. R. Harrison, and hydroxytamoxifen (HF) was obtained from Schering-Plough Corp.

Modeling—The crystal structure of the hAR LBD bound to R1881 was used as the starting model (17). R1881 was replaced by E2 in the model using SPDBV. Mutations in the structure were introduced using the SWISS-MODEL (18). The resulting models were energy-minimized using the CNS suite of programs (19) with the parameter and topology files for the ligands obtained from the HIC-Up server (x-ray.bmc.uu.se/hicup) (20).

Site-directed PCR Mutagenesis—The positions of AR mutations are based on the presumed E2 contact sites determined via the molecular model of AR bound with E2, considering residues within 4.5 Å of E2 (Fig. 1, A and B). The AR residues were changed to the homologous residue of ER (Fig. 1B). The AR/M749I prostate cancer and AR/M749V androgen insensitivity phenotype mutations (8) were also constructed using site-directed mutagenesis. In each case, the presence of the correct mutation was confirmed by sequencing.

Cell Culture, Transfections, and Reporter Gene Expression Assays—AR-negative DU145 human prostate cancer cells and both AR- and ER-negative COS-1 monkey kidney cells were maintained in phenol red-free Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate with 5% CO2 at 37 °C. Transfections and luciferase assays were performed as previously described (12). pCMV-β-galactosidase was used as an internal control. Data are presented as the mean ± S.D. of at least three independent experiments.

Steroid Binding Assay—Steroid binding was performed on intact COS-1 cells as described previously (12). COS-1 cells were transfected with either the wtAR or the mutant (5 μg). Forty-eight hours after transfection, the cells were treated with [3H]E2, ranging from 0.01 to 1 nM in the presence or absence of a 100-fold molar excess of unlabeled E2, for 2 h, at 37 °C, in a 5% CO2 incubator. Specific binding was determined from the difference between radioactivity in the presence or absence of unlabeled E2. Scatchard plot analysis was used to determine binding affinity. Data are presented as a mean ± S.D. of three independent experiments.

E2 Dissociation Assay—The whole cell-binding assay was used to determine the effect of mutation on dissociation of E2 from the AR (12). COS-1 cells were transfected with either the wtAR or the mutant (5 μg). After 24 h, the transfected cells were treated with 1 nM [3H]E2 in the presence or absence of 1000-fold excess of unlabeled E2, for 37 °C, in a 5% CO2 incubator. Specific binding was determined from the difference between radioactivity in the presence or absence of unlabeled E2. Scatchard plot analysis was used to determine binding affinity. Data are presented as a mean ± S.D. of three independent experiments.

Limited Trypsinization Assay—In vitro transcription/translation reactions were performed using the Tnt-coupled reticulocyte lysate system (Promega) in the presence of [35S]methionine. Two microliters of labeled translation mixture were incubated for 30 min at room temperature, with 2 μl of ethanol, 10 nM DHT, or 100 nM E2. Limited trypsinization was performed by addition of 1 μl of trypsin solution (50 μg/ml) (dissolved in 50 mM Tris HCl pH 7.5, 1 mM CaCl2) for various lengths of time, at 25 °C. Reactions were stopped by addition of 1 μl of SDS sample buffer, and samples were separated by 11% SDS-PAGE. Autoradiography was performed overnight.

Data Analysis—Pooled data are reported as the mean ± S.D., and statistical significance was determined using the Student’s unpaired t test. Probabilities < 5% (p < 0.05) were considered significant.

RESULTS

Molecular Model of the AR Ligand Binding Domain Bound to E2—The molecular models of the AR-LBD bound to either E2 (Fig. 1) or DHT (data not shown) were constructed based on the crystal structure of the human AR-LBD with R1881, a synthetic androgen, as the bound ligand (17). The model shows the selected residues of AR that are predicted to be within 4.5 Å of E2. B, list of residues identified as E2 contact sites in the AR-LBD pocket. Columns shows AR-LBD residues (column 1), corresponding ER-LBD residues (column 2), either ligand, DHT or E2, that contacts each AR-LBD residue (column 3), and the AR helix containing each AR-LBD residue (column 4). Among these residues, Leu-704, Gly-708, Gin-711, Met-742, Met-745, Met-749, Arg-752, Phe-764, Met-787, Leu-976, and Thr-877 are identified as the contact sites for R1881 in the AR crystal structure (16, 20). D, DHT; E, E2.

Fig. 1. E2 contact sites of the AR-LBD pocket. A, the molecular model of E2 bound to the AR-LBD was constructed based on the crystal structure of the human AR-LBD with R1881, a synthetic androgen, as the bound ligand (17). The model shows the selected residues of AR that are predicted to be within 4.5 Å of E2. B, list of residues identified as E2 contact sites in the AR-LBD pocket. Columns shows AR-LBD residues (column 1), corresponding ER-LBD residues (column 2), either ligand, DHT or E2, that contacts each AR-LBD residue (column 3), and the AR helix containing each AR-LBD residue (column 4). Among these residues, Leu-704, Gly-708, Gin-711, Met-742, Met-745, Met-749, Arg-752, Phe-764, Met-787, Leu-976, and Thr-877 are identified as the contact sites for R1881 in the AR crystal structure (16, 20). D, DHT; E, E2.
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Methionine 749 Is a Hot Spot for Mutation in AR-associated Diseases.

A. Methionine (Met) at AR position 749 is mutated to isoleucine (Ile) in androgen-independent prostate cancer (22) and to valine (Val) in complete or partial androgen insensitivity syndrome (AIS) (23, 24). An AR mutant in which amino acid 749 is changed from Met to Leu has not yet been identified to have linkage with AR-associated diseases. B, side-chain differences among amino acid residues at position 749 in AR mutants.

Fig. 3. AR(M749L) is a mutation hot spot for AR-associated diseases. A, methionine (Met) at AR position 749 is mutated to isoleucine (Ile) in androgen-independent prostate cancer (22) and to valine (Val) in complete or partial androgen insensitivity syndrome (AIS) (23, 24). An AR mutant in which amino acid 749 is changed from Met to Leu has not yet been identified to have linkage with AR-associated diseases. B, side-chain differences among amino acid residues at position 749 in AR mutants.

Fig. 2. AR(M749L) confers hypersensitivity to E2. A, a series of AR mutants were screened in the presence of E2. AR-negative DU145 cells were transfected with 3.5 μg of (ARE)4-pG1-luciferase, 10 μg of pCMV-β-galactosidase internal control plasmid, and 1.5 μg of either the wtAR or an AR mutant. Transfected cells were treated with ethanol (as a control) or 1 or 10 nM E2 for 24 h. The CWR22:AR(H874Y) mutant construct was used as a positive control for estrogen stimulation (14). Luciferase activities were normalized according to β-galactosidase activities and are expressed as -fold induction relative to that with ethanol treatment. The values represent the mean ± S.D. from three independent assays.

B, transactivation profile of the wtAR, AR(M749L), and AR(H874Y) in the presence of DHT. DU145 cells were transfected with 3.5 μg of (ARE)4-pG1-luciferase, 10 μg of pCMV-β-galactosidase internal control plasmid, and 1.5 μg of wtAR, AR(M749L), AR(H874Y), or various other AR mutants. Transfected cells were treated with ethanol (as a control) or 1 or 10 nM DHT for 24 h. AR transactivation in response to DHT was used to confirm the functional ability of the AR(H874Y) construct. Weak E2 stimulation of AR(H874Y) in DU145 cell, in contrast with previous studies using CV-1 cells (14), may be due to cell-specific effects. Luciferase activities were normalized according to β-galactosidase activity and are expressed as -fold induction relative to ethanol treatment. The values represent the mean ± S.D. from three individual assays.

Gly-708, Gln-711, Met-742, Met-745, Arg-752, Phe-764, Met-787, Leu-873, and Thr-877 are not only the contact sites identified by the molecular model of AR-LBD bound to E2 but also the contact sites in the molecular model of the AR-LBD bound to DHT (data not shown). With the exception of residue Leu-707, the same residues have been identified as the contact sites of R1881 in the AR-LBD crystal structure (16, 17, 21). Conserved amino acids in the LBDs of AR and ER, Leu-704, Leu-707, Arg-752, and Phe-764 indicate the importance of these residues for the receptor-LBD pocket structure. Val-746 and Leu-873 have been identified as the primary E2 contact sites in the AR-LBD.

AR(M749L) Mutant Confers Hypersensitivity to E2—To isolate an E2-sensitive AR mutant, we used site-directed mutagenesis to introduce several point mutations over 14 residues in the wtAR-LBD. The positions of mutations are based on the E2 contact sites in the E2-AR molecular model (Fig. 1A). The AR contact site residues were replaced with the corresponding ER residues (Fig. 1B), and the desired mutations were confirmed by sequencing. A luciferase reporter assay was used to screen the AR mutants for their transactivation under 1 or 10 nM E2.

Fig. 3 (A and B) demonstrates that wtAR, the AR(M749L) mutant, and the AR(H874Y) mutant are induced to approximately the same level in response to DHT. This result confirms that the AR(H874Y) plasmid is functional and suggests that there are cell-specific factors that mediate differences in E2 induction of AR mutant in CV-1 versus DU145 cells. Nevertheless, based on Fig. 2A and B, we can conclude that the AR(M749L) mutant is highly sensitive to E2 using the (ARE)4-pG1-luciferase reporter construct in DU145 cells. The AR(7708A), like wtAR, can be activated in response to DHT, but not to E2 (Fig. 2A, lanes 5 and 6) versus B (lane 4). The AR(Q711E), AR(M742L), AR(M745L), AR(M787L), AR(L873G), and AR(T877L) are very weakly activated by DHT. We also tested the expression level of each mutant and found similar expression levels of the AR mutants compared with wtAR (data not shown).
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Fig. 4. AR(M749L) responds to 0.1 nM E2.—A, DU145 cells were transfected with 3.5 µg of (ARE)4-pG1-luciferase, 10 ng of pCMV-β-galactosidase internal control plasmid, and 1.5 µg of either wtAR or an AR mutant. Transfected cells were treated with either ethanol (as a control) (lanes 1 and 7), various concentrations of DHT at 0.001, 0.01, 0.1, 1, or 10 nM (lanes 2–6), or various concentrations of E2 at 0.001, 0.01, 0.1, 1, or 10 nM (lanes 8–12) for 24 h. Luciferase activities were normalized according to the β-galactosidase activity and are expressed as -fold induction compared with ethanol treatment. The values represent the mean ± S.D. from three individual assays. B, DU145 cells were transfected with 3.5 µg of either the (ARE)-PSA-luciferase or the (ARE)2-MMTV-luciferase reporter, 10 ng of pCMV-β-galactosidase internal control plasmid, and 1.5 µg of the wtAR, AR(M749L), AR(M749I), or AR(M749V). Transfected cells were treated with either ethanol (as a control) (lanes 1 and 6), 1 nM DHT (lanes 2 and 7), 10 nM DHT (lanes 3 and 8), 1 nM E2 (lanes 4 and 9), or 10 nM E2 (lanes 5 and 10) for 24 h. Luciferase activities were normalized according to the β-galactosidase activity and are expressed as -fold induction compared with ethanol treatment. The values represent the mean ± S.D. from three individual assays.

The AR(M749L) mutant shows clear activation at 10 nM DHT. However, in contrast to AR(M749L), the lower sensitivity of AR(M749V) at the physiological concentration of 1 nM DHT matches its association with the AIS phenotype. In terms of sensitivity to estrogen, AR(M749V) also demonstrated an estrogenic profile, similar to AR(M749L), with lower intensity and shows higher sensitivity than wtAR in response to 10 nM E2 (lane 12). The AR(M749I) mutant shows very weak activation in response to 10 nM DHT (lane 6) and a negligible level of activation in response to 10 nM E2 (lane 12).

We also made use of two AR target gene (PSA and MMTV) promoter reporters, to confirm the responsiveness of AR(M749L), AR(M749I), and AR(M749V) to estrogen. Fig. 4B shows not only high stimulation of AR(M749L) (lanes 4 and 5, and 9 and 10) in response to E2, but also the relative stimulation of AR(M749V) by E2 (lanes 4 and 5, and 9 and 10), which are effects that can be demonstrated consistently with different reporters. The effects of DHT are less consistent among the mutants using various reporter genes. AR(M749V)-mediated induction in response to 1 nM DHT is higher using the PSA reporter construct. 10 nM DHT weakly induced AR(M749I) transactivation of both the (ARE)-PSA (lane 3) and (ARE)2-MMTV (lane 8) promoters, however, E2 did not significantly induce transactivation of AR(M749I) using either of the promoters (lanes 4, 5, 9, and 10). Together, the data indicate that Met-749 of AR is a critical amino acid involved in the control of ligand specificity and that particular mutants of Met-749 intensify the responsiveness of AR to E2.

AR(M749L) Strongly Responds to E2 but Not to Progesterone or Dexamethasone—Fig. 5A shows the sequence alignment and
homology of the helix 5 region of the human AR (hAR), human progesterone receptor (hPR), human GR (hGR), and human ERα (hERα) is shown (21). The sequence position is given for the hAR (top) and hERα (bottom). Residues conserved among steroid receptors are in italics. Identical residues among the aligned receptor sequences are shaded. The residue of focus in this study, Met-749, is highlighted in boldface for comparison with amino acids at the same position in other steroid receptors. At position 749, hAR contains a methionine (M) residue, whereas the same residue in hPR, hGR, and hERα, after alignment, is leucine (L). B, the luciferase activity of a receptor gene in response to the wtAR, AR(M749L), AR(M749I), and AR(M749V) upon treatment with 10 nM DHT, E2, progesterone, or dexamethasone. COS-1 cells were transfected with 3.5 µg of the (ARE)4-pG1-luciferase reporter, 10 ng of pcMV-β-galactosidase internal control plasmid, and 1.5 µg of either the wtAR, AR(M749L), or AR(M749V). Transfected cells were treated with either ethanol (as a control) (lanes 1, 6, 11, and 16), 10 nM DHT (lanes 2, 7, 12, and 17), 10 nM E2 (lanes 3, 8, 13, and 18), 10 nM progesterone (lanes 4, 9, 14, and 19), or 10 nM dexamethasone (lanes 5, 10, 15, and 20) for 24 h. Luciferase activities were normalized according to β-galactosidase activity and are expressed as -fold induction relative to ethanol treatment. The values represent the mean ± S.D. from three individual assays.

one-third of the induction mediated by E2 (lane 8). Induction of reporter activity stimulated by progesterone is not surprising, because hPR and hAR share the greatest structural homology among the classic steroid receptors. 10 nM dexamethasone did not stimulate AR(M749L) transactivation (lane 10) indicating that the position of the AR mutation, M749L, is particularly important for E2 stimulation. AR(M749V) is clearly activated by 10 nM DHT (lane 17) and weakly activated by 10 nM E2 (lane 18), compared with ethanol treatment (lane 16), similar to the induction of AR(M749V) activity by 10 nM DHT and 10 nM E2 in DU145 cells (Fig. 4). Neither 10 nM progesterone nor 10 nM dexamethasone stimulated AR(M749V) transactivation (lanes 19 and 20), and AR(M749I) shows negligible activation in response to 10 nM progesterone or dexamethasone (lanes 14 and 15).

E2-induced AR(M749L) transactivation is via the AR not the ER. AR-negative, ER-negative COS-1 cells were transfected with 3.5 µg of the (ARE)4-pG1-luciferase reporter, 10 ng of pcMV-β-galactosidase internal control plasmid, and 1.5 µg of either the wtAR or an AR mutant. Transfected cells were treated with ethanol as a control (lane 1) or with the various combinations of ligand and antiandrogen as indicated. E2 stimulation of AR(M749L) and AR(M749V) in COS-1 cells supports the data obtained in DU145 prostate cancer cells, shown in Fig. 4, demonstrating the lack of cell-specific effects. No significant wtAR activity was detected at the supra-physiological concentration of 100 nM E2 (lane 10). The high concentration of 100 nM E2 was used to demonstrate the clear blocking effects of antiandrogens. Luciferase activities were normalized according to β-galactosidase activity and are expressed as -fold induction compared with ethanol treatment. The values represent the mean ± S.D. from three individual assays.

The sequence alignment indicates that all steroid receptors contain Leu at amino acid 749, except AR, which contains Met at that position. We next tested whether the broadened specificity of AR(M749L) involves only E2 or applies to other steroids as well. Fig. 5B shows reporter transactivation mediated by the wtAR, AR(M749L), AR(M749I), and AR(M749V) in the presence of 10 nM DHT, 10 nM E2, 10 nM progesterone, or 10 nM dexamethasone. The wtAR is activated by 10 nM DHT (lane 2) but not by other steroids (lanes 3–5), compared with no activation upon ethanol treatment (lane 1). AR(M749L) is significantly activated by 10 nM DHT (lane 7) and 10 nM E2 (lane 8), is moderately induced by 10 nM progesterone (lane 9), but shows no induction with 10 nM dexamethasone (lane 10), compared with no activation upon ethanol treatment (lane 6). 10 nM E2 stimulates AR(M749L) activity (lane 8) to a higher level compared with 10 nM DHT treatment (lane 7), in COS-1 cells. The reporter induction mediated by progesterone (lane 9) is
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Analysis of equilibrium binding of [3H]E2 to wtAR and AR mutant, molar excess of unlabeled E2 with continued incubation at 37°C measured. The values are the means ± S.D. of three individual assays.

COS-1 cells were harvested at the indicated times, and the radioactivity was measured. The values are the means ± S.D. of three individual assays.

Mutants induced by E2 occurs via the AR. The AR- and ER-negative COS-1 cells were used for transfection. Antiandrogens, such as HF and ICI 176,334 (casodex or bicalutamide), were used to block transactivation of the AR mutants in response to E2. If E2 induction of wtAR is inhibited by addition of antiandrogens, it indicates that E2 action occurs through AR.

To demonstrate the antiandrogenic activity of HF and ICI 176,334 on AR-mediated transactivation in the presence of DHT or E2, transfected cells were treated with different combinations of DHT or E2 with HF or ICI 176,334. Induction of the wtAR and AR(M749V) mutant was negligible, whereas there was moderate induction of the AR(M749L) mutant in the presence of 1 nM DHT and 5 µM E2 (Fig. 6, lane 6). This partial antagonistic effect of HF on AR(M749L) is reasonable in that HF itself has been shown to stimulate AR(M749L) (lane 2). The transactivation of all three receptors, wtAR, AR(M749L), and AR(M749V), was also suppressed in a dose-dependent manner in the presence of 1 µM (lane 7) or 10 µM (lane 8) ICI 176,334.

E2-induced AR(M749L) transactivation was detected at both 10 and 100 nM E2 (lanes 9 and 10), whereas AR(M749V) transactivation was strongly induced only at 100 nM E2, E2 stimulation of AR(M749L) and AR(M749V) in COS-1 cells supports the data obtained in DU145 prostate cancer cells, shown in Fig. 4, demonstrating the lack of cell-specific effects. No significant wtAR activity was detected upon treatment with the supraphysiological concentration of 100 nM E2 (lane 10). The high concentration of 100 nM E2 was used to demonstrate the clear blocking effects of antiandrogens. Repressive effects of HF on E2-induced AR(M749L) and AR(M749V) transactivation were detected (lane 11). Also, dose-dependent responses of the AR mutants to ICI 176,334 were observed when the antiandrogen was combined with various concentrations of E2 (lanes 12–15). Similar profiles of antiandrogen blockade of DHT or E2-induced wtAR or AR mutant transactivation were observed when using DU145 prostate cancer cells (data not shown). HF and ICI 176,334 can antagonize E2 induction of AR(M749L) and AR(M749V) and therefore indicate that E2 modulates AR function directly.

AR(M749L) Slows E2 Dissociation from AR—To test whether E2 stimulation of AR mutants could be due to the improved

![Fig. 7. AR(M749L) retards E2 dissociation. A, Scatchard plot analysis of equilibrium binding of [3H]E2 to wtAR and AR mutant, AR(M749L), was determined in whole cell binding assays as described (12). COS-1 cells were transfected with either the wtAR or the mutant (5 µg) and treated with 0.1–1 nm [3H]E2 in the presence or absence of 100-fold excess unlabeled E2 for 2 h at 37 °C. The values represent the means ± S.D. of three individual assays. B, counts (cpm) of [3H]E2 bound in the absence of unlabeled E2 competitor to wtAR, AR(M749L), AR(M749L), or AR(M749V). C, counts (cpm) of [3H]E2 bound to the wtAR, or AR(M749L), in the presence of excess unlabeled E2. The wtAR or AR mutant (5 µg) were transiently expressed in COS-1 cells. Transfected cells were treated with 1 nm [3H]E2 for 2 h, at 37 °C. After 2 h, the dissociation was initiated with the addition of a 1000-fold molar excess of unlabeled E2 with continued incubation at 37 °C. The cells were harvested at the indicated times, and the radioactivity was measured. The values are the means ± S.D. of three individual assays. D, Luciferase activity (Relative unit) stable transfection of the AR, the combination of E2 and DHT increased AR(M749L) transactivation. COS-1 cells were transfected with 3.5 µg of the AR(M749L)-luciferase reporter, 10 ng of pCMV-β-galactosidase internal control plasmid, and 1.5 µg of either the wtAR or an AR mutant. Transfected cells were treated with ethanol as a control (lanes 1, 7, and 13), 0.01 nM DHT (castration or very low level) (lanes 2, 8, and 14), 1 nM DHT (lanes 3, 9, and 15), 10 nM E2 (lanes 4, 10, and 16), the combination of 0.01 nM DHT plus 10 nM E2 (lanes 5, 11, and 17), or 1 nM DHT plus 10 nM E2 (lanes 6, 12, and 18). Luciferase activities were normalized to β-galactosidase activity and are expressed as fold induction compared with the basal activity upon ethanol treatment. The values represent the mean ± S.D. from two or more assays.

*p < 0.05, significant decrease in [3H]E2 dissociation from the AR(M749L) compared with the wtAR. D, the combination of E2 and DHT increased AR(M749L) transactivation. COS-1 cells were transfected with 3.5 µg of the AR(M749L)-luciferase reporter, 10 ng of pCMV-β-galactosidase internal control plasmid, and 1.5 µg of either the wtAR or an AR mutant. Transfected cells were treated with ethanol as a control (lanes 1, 7, and 13), 0.01 nM DHT (castration or very low level) (lanes 2, 8, and 14), 1 nM DHT (lanes 3, 9, and 15), 10 nM E2 (lanes 4, 10, and 16), the combination of 0.01 nM DHT plus 10 nM E2 (lanes 5, 11, and 17), or 1 nM DHT plus 10 nM E2 (lanes 6, 12, and 18). Luciferase activities were normalized to β-galactosidase activity and are expressed as fold induction compared with the basal activity upon ethanol treatment. The values represent the mean ± S.D. from two or more assays.
ability of AR to bind E2, COS-1 cells were transfected with either the wtAR or the mutant. The affinity of wtAR or a mutant for E2 was measured by the binding assay, using [3H]E2 in the presence or absence of a 100-fold excess unlabeled E2. After performing Scatchard analysis, the equilibrium binding affinity for [3H]E2 determined by the whole cell binding assay was not found to be altered significantly in AR(M749L) compared with wtAR (Fig. 7A). Therefore, the binding ability of E2 does not significantly contribute to the E2-dependent AR(M749L) transactivation.

We further tested whether the AR mutations influence the dissociation of E2 from AR. Fig. 7B represents the [3H]E2 binding affinity of various ARs labeled with 1 nM [3H]E2 without unlabeled E2 competitor, to be used as a control. Fig. 7C represents [3H]E2 counts of AR wt, or the AR(M749L) mutant, after 2 h of labeling with [3H]E2 followed by initiation of dissociation by changing the medium to that which contains a 1000-fold excess of unlabeled E2. The reduction in bound counts after addition of excess E2 demonstrates the [3H]E2 dissociation. We have performed this assay over extended periods, including 1, 2, 3, 4, 5, and 8 h in three independent assays, because the later time points are more relevant and a more prolonged effect on ligand dissociation will have a greater influence on AR transactivation. Fig. 7C shows that over time there was a pronounced retardation in [3H]E2 dissociation from AR(M749L), compared with the wtAR, in the presence of excess unlabeled E2. There was no statistically significant difference in the [3H]E2 dissociation between the wtAR and AR(M749I) or AR(M749V) (data not shown).

Additive Induction of AR(M749L) with Combined E2 and DHT Treatment—We next tested the combined effect of DHT and E2 on wtAR, AR(M749L), and AR(M749V) transactivation. Fig. 7D shows weak (lane 2) and strong (lane 3) activation of wtAR detected in the presence of 0.01 and 1 nM DHT, respectively. Reduced activation of wtAR was detected in the presence of either 0.01 nM (castration or low level) or 1 nM DHT and 10 nM E2 (lanes 5 and 6). We repeated this experiment in DU145 cells and confirmed the antagonistic effect of 10-fold excess E2 on DHT-induced AR transactivation (data not shown). AR(M749L) shows a very weak response to 0.01 nM DHT (castration level) in COS-1 cells (lane 7 versus 8). In contrast, significant levels of AR(M749L) activation were detected in the presence of both E2 and DHT (lanes 11 and 12) compared with transactivation induced by either DHT or E2 alone (lanes 8–10). Similar profiles were detected with the AR(M749V) mutant (lanes 17 and 18 versus lanes 14 and 15), but at lower activity levels. The differential induction of wtAR versus AR(M749L), after combining DHT and E2 treatment, could be explained by the existence of different ligand saturation thresholds of the wtAR compared with AR(M749L). E2 at 10-fold excess may compete with DHT for binding to wtAR and result in repression of DHT-induced wtAR transactivation. In the case of the AR mutants, DHT may not be able to saturate the receptors, and the addition of E2 enhances receptor transactivation. Therefore, the AR(M749L) mutation may allow more efficient and/or productive ligand binding for both DHT and E2.

AR(M749L)-bound E2 Resists Trypsin Digestion—To understand the different effects of E2 on wtAR and AR(M749L) transactivation, in vitro translated wtAR or AR(M749L) was incubated in the presence or absence of ligands. Limited trypsinization was performed for various lengths of time and proteolysis-resistant fragments were analyzed. Fig. 8A shows the expression levels of wtAR (lane 1) and AR(M749L) (lane 2). Fig. 8B shows a 29-kDa proteolysis resistant fragment of both wtAR and AR(M749L), which formed in the first 10 min of incubation with trypsin after addition of 10 nM DHT. This is consistent with previous studies (26). In the absence of ligand, the wtAR and AR(M749L) were completely degraded (lanes 1 and 13). However, we can see the 29-kDa proteolysis-resistant fragment of AR(M749L) after addition of 100 nM E2 (lane 15), but a resistant wtAR fragment is not visible (lane 3). As incubation time is increased, another small fragment (20-kDa) becomes apparent and the 29-kDa fragment begins to disappear (lanes 18 and 21). This suggests that E2-wtAR, resulting in differences in resistance to trypsin digestion, or alternatively, E2-AR(M749L) takes on a different conformation than E2-wtAR, resulting in differences in resistance to trypsin digestion, or alternatively, E2-AR(M749L) takes on a conformation similar to DHT-wtAR resulting in a trypsin digestion pattern resembling that of DHT-wtAR.

AR(M749L) Influences Coregulator Modulation of AR Transactivation—We next tested whether AR mutations affect coregulator modulation of receptor transactivation in response to either 10 nM DHT, 10 nM E2, or 100 nM ICI 176,334. In fact, AR(M749I) was identified as a somatic mutation in androgen-independent prostate cancer, which often results from combined treatment involving total androgen withdrawal and treatment with the antiandrogen drug bicalutamide (27). Therefore, we tested whether the AR(M749I) and AR(M749L) mutations enable the antagonist ICI 176,334 to become an agonist in the presence of coregulators.

AR-negative DU145 cells were transfected with 2.5 μg of (ARE)4-pG1-luciferase reporter and 1 μg of various forms of AR in the presence or absence of 3 μg of ARA70 or SRC-1. The parent pSG5 vector (3 μg) was used to substitute for pSG5-ARA70 or pSG5-SRC-1 in the AR alone transfection. Luciferase activity was normalized according to β-galactosidase activity, and the -fold luciferase activity is expressed as the -fold induction relative to ethanol treatment (set as 1-fold). Fig. 9 shows that coregulators of the AR, ARA70 and SRC-1, significantly promoted DHT- or E2-dependent transactivation of the AR.
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(749L) (lanes 5, 6, 8, and 9). SRC-1 promoted E2-dependent AR(M749L) transactivation to a higher level than ARA70 and compared with that of the wtAR (lanes 9 versus 8). However, there is negligible activation of the wtAR, AR(M749L), or AR(M749I) in response to 100 nM ICI 176,334, even with overexpression of ARA70 or SRC-1 (lanes 11 and 12).

Analysis of the Effect of Met-749 in the AR-LBD Pocket—To understand the effects of AR mutations, we examined the locations of various mutated residues in the AR-LBD crystal structure. For our examination, we replaced the R1881 model with the E2 model (Fig. 10) and minimized the energy of the resulting structure. We also changed the identity of residue 749 from Met to Leu or Val and minimized the energy of the resulting structures. In all cases, energy minimization yielded final energies that are very similar and did not greatly perturb the AR structure. Modeling is still at an insufficient stage of development for us to reliably discern which small-scale shifts in the structure might be of actual significance. However, absence of an increase in the model energy indicates that the AR structure is easily able to accommodate these changes. The sole mutation that has an effect on E2-induced transactivation occurs at position 749. As shown in Fig. 10, this residue forms the wall of the binding pocket that is close to the O3 atom of the ligand. The O3 atom is bonded to the A ring of the ligand and happens to be at one of the regions where the difference between E2 and DHT is greatest. In E2, the O3 is part of a hydroxyl group, and the A ring is aromatic (planar) in structure. In DHT, the O3 atom is a keto oxygen, and the A ring is not aromatic and adopts a chair conformation instead. Thus, the location of a mutation that confers E2 hypersensitivity is near one of the areas of significant difference between E2 and DHT. Such positioning provides compelling corroborating evidence that this residue is critical for discriminating between DHT and other ligands in the process of transactivation.
The residues of the ligand contact site of AR are hypothesized to be responsible for the proper interaction and packaging of the ligand within the receptor LBD pocket (27). The ligand selectivity does not depend on the number of hydrophobic contacts made by the ligand within the large LBD pocket (27) but rather on how the ligand interacts with specific key contact sites within the pocket. After a ligand binds to the receptor, the conformational change induced allows the recruitment of cellular coregulators and the initiation of AR transactivation. Although $E_2$ could bind to the wtAR (Fig. 7) (12), it was unable to induce wtAR transactivation. There are several possible reasons for the inability of $E_2$ to stimulate AR activity: 1) $E_2$ may not bind properly within the wtAR-LBD pocket, 2) weakly bound $E_2$ may easily escape from the wtAR-LBD pocket, and 3) the $E_2$-AR complex may not adopt the proper conformation necessary for AR transactivation.

We were interested in isolating an AR mutant that is hypersensitive to $E_2$, to study how estrogen may regulate androgen targets. The $E_2$-bound AR modeling approach was used primarily to identify residues that might affect ligand binding specificity and ligand-specific activation. As shown in Figs. 1, 2, 4, and 5, Met-749 is the most influential residue of the $E_2$ contact site within the AR-LBD pocket and is important for $E_2$ regulation of AR function. The functional data show that AR(M749L) has two significant characteristics: 1) it does not significantly diminish DHT activation, suggesting that position 749 is not critical for DHT induction, and 2) upon $E_2$ binding, the AR(M749L) mutant is hypersensitive to $E_2$ stimulation, suggesting that the residue identity at position 749 is a major determinant in allowing $E_2$-mediated AR induction. As shown in Figs. 7–9, $E_2$-dependent AR(M749L) transactivation may be dependent upon the length of time $E_2$ is able to remain locked into the AR-LBD, providing the preferred conformation necessary for coregulator recruitment and achievement of the final steps of AR transactivation. Fig. 7C demonstrates that $E_2$ dissociates more slowly from the AR(M749L) LBD pocket than from the wtAR, suggesting that length of ligand association is a crucial factor in induction of transactivation. There is no clear correlation observed between the ligand binding affinity and the functional activity of AR(M749L), with strong induction of transactivation observed in response to $E_2$. Such an effect is similar to previous studies reporting that the binding properties and relative biological potencies of a number of steroids do not always correlate (12, 15). Furthermore, Fig. 7D shows that combined DHT and $E_2$ structure increases the activity of AR(M749L) compared with the response to DHT alone. In contrast, the activation of the wtAR by DHT is reduced in the presence of $E_2$, suggesting that there is competition between DHT and $E_2$ for binding to the wtAR. The additive effect of DHT and $E_2$ in the activation of AR(M749L) indicates the possibility of differential structural conformation between AR(M749L) and wtAR, with that of AR(M749L) allowing more efficient and/or productive binding of both DHT and $E_2$. Differences in the susceptibility of the wtAR and AR(M749L) to proteolysis in the presence of $E_2$ are demonstrated in Fig. 8, indicating that the conformation of $E_2$-bound AR(M749L) is different from that of $E_2$-bound wtAR, as well as the existence of complex specific coregulator recruitment. In parallel, Fig. 9 shows that coregulators of AR, such as AR70 and SRC-1 (10, 12), significantly increase DHT- or $E_2$-dependent AR(M749L) transactivation compared with wtAR transactivation, and these data indicate that DHT- or $E_2$-bound AR(M749L) produces an active interface for coregulator interaction, resulting in modulation of transactivation. SRC-1 promoted $E_2$-dependent AR(M749L) transactivation to a higher level than did AR70, suggesting a preferential $E_2$-dependent modulation effect by SRC-1. It is also possible that SRC-1 is better able to relieve repression by one or more AR-bound corepressors than is AR70. Therefore, AR(M749L) harbors changes in the LBD that not only permit AR(M749L) to accept $E_2$ but also allow the conformational changes that allow the modulation by coregulators that is necessary for AR(M749L) transactivation. Mutation of Met-749 to Leu leads to 1) retardation of $E_2$ dissociation from the mutant receptor pocket, 2) adoption of an active conformation, and 3) recruitment of the modulators necessary for the induction of $E_2$-induced AR(M749L) activity.

In terms of steroid structure, there is a significant difference between $E_2$ and DHT in the A ring of each molecule (Fig. 10A). The A ring is aromatic in $E_2$ but not in DHT. Residue 749 of AR abuts the A ring of a steroid when it is bound. It is possible that the Met residue at this position allows DHT binding and resultant receptor transactivation while limiting access to $E_2$. The mutation of Met-749 to Leu or Val reduces this selectivity and allows both DHT and $E_2$ to activate AR. Although both Leu and Val have similar side-chain characteristics, the differential effects of Leu versus Val in the intensity of $E_2$-dependent transactivation is not readily explained in structural terms by examining the crystal structure of the AR-ligand complex or by simple modeling exercises. The other major difference between DHT and $E_2$ structure is the presence of a methyl group in DHT (C19). When DHT is bound by AR, the only side chain within the van der Waals distance of the C19 methyl group is that of Gln-711. A mutation of the Gln-711 residue may affect the proper accommodation of $E_2$ in the AR-LBD pocket. In our experiments, we screened a mutant wherein glutamine, Gln-711, had been changed to glutamic acid, Glu, which does not result in responsiveness to $E_2$ (Fig. 2A) but instead mediates a loss of responsiveness to DHT (Fig. 2B).

It is also interesting that, as shown in Fig. 3, different amino acid substitutions at Met-749 are associated with various AR-related diseases, including prostate cancer and complete or partial AIS. AR(M749I) was identified as a somatic mutation in androgen-independent prostate cancer, which often results from combined treatment involving total androgen withdrawal and the antiandrogen drug bicalutamide (28). The AR(M749I) mutation disrupted the responsiveness of AR to DHT and $E_2$, and, although $E_2$ may play a role in androgen-independent prostate cancer progression, there is no influence of $E_2$ or other steroids, such as progesterone or dexamethasone, on AR(M749I) transactivation. Furthermore, overexpression of coregulators did not increase AR(M749I) transactivation. Therefore, androgen-independent prostate cancer progression in patients with this AR mutation may be mediated via alternate signaling pathways rather than resulting from reduced ligand specificity of AR(M749I).

Another naturally occurring AR mutant, AR(M749V), has been linked to the AIS phenotype, a condition characterized by loss of androgen action in male development. AR(M749V) is sufficiently activated in response to 10 nM DHT but not by 1 nM DHT, the physiological concentration of androgen. This weak response of AR(M749V) to physiological androgen levels may be the cause of the defective AR signaling that leads to the development of androgen insensitivity. AR(M749V) is activated to a higher level by 10 nM $E_2$ than is wtAR, but this stimulation does not reach the level of wtAR induction by DHT. Therefore, $E_2$-AR(M749V) signaling could not compensate for the insufficient DHT-AR(M749V) signaling in AIS patients.

In summary, AR(M749L), a mutant that responds to the agonistic effect of $E_2$ in a manner similar to the response of wtAR to DHT, may be a useful tool with which to study the influence of estradiol on AR function. Additional characteriza-
tion of this hyperestrogenic AR(M749L) mutant will also yield information regarding the influence of estrogen on particular androgen targets.

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