Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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30. Protein kinase assays were performed in 25 μl of kinase buffer (*4*) with 80 mM KCl (Fig. 3B) or without KCl (Fig. 3, A and C) and containing 0.1 mM [γ-^{32}P]ATP (8000 cpm/pmol), 2.4 μg of Cph1-PCB adduct or 1 μg of N514-PCB adduct in Pr or Pfr form, and 2 μg of MBP-Rcp1 (WT and D68A). Reactions were initiated by adding ATP, mixtures were incubated 30 min at 30°C, and reactions were stopped by adding SDS sample buffer (*4*).
31. We are indebted to E. Campbell for providing us with purified *Synechocystis* DNA, K. C. McFarland for figure design, A. Skerra for core streptavidin, and D. Kehoe for critical review of the manuscript. Supported in part by National Science Foundation grant MCB-9604511 to J.C.L. and NIH National Center for Research Resources award 1 P41 RR06009 to the Pittsburgh Supercomputing Center.

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Differential Ligand Activation of Estrogen Receptors ERα and ERβ at AP1 Sites

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The transactivation properties of the two estrogen receptors, ERα and ERβ, were examined with different ligands in the context of an estrogen response element and an AP1 element. ERα and ERβ were shown to signal in opposite ways when complexed with the natural hormone estradiol from an AP1 site: with ERα, 17β-estradiol activated transcription, whereas with ERβ, 17β-estradiol inhibited transcription. Moreover, the antiestrogens tamoxifen, raloxifene, and Imperial Chemical Industries 164384 were potent transcriptional activators with ERβ at an AP1 site. Thus, the two ERs signal in different ways depending on ligand and response element. This suggests that ERα and ERβ may play different roles in gene regulation.

Antiestrogens are therapeutic agents for the treatment and possible prevention of breast cancer. Tamoxifen (Fig. 1A) is an antiestrogen that is used in breast cancer chemotherapy and is believed to function as an antitumor agent by inhibiting the action of the estrogen receptor (ER) in breast tissue (*1*). Paradoxically, tamoxifen appears to function as an estrogen-like ligand in uterine tissue, and this tissue-specific estrogenic effect may explain the increased risk of uterine cancer that is observed with prolonged tamoxifen therapy (*2*). The related benzothiophene analog raloxifene (Fig. 1A) has been reported to retain the antiestrogen properties of tamoxifen in breast tissue and to show minimal estrogen effects in the uterus; in addition, it has potentially beneficial estrogen-like effects in nonreproductive tissue such as bone and cardiovascular tissue (*3*–*7*). One explanation for these tissue-specific actions of antiestrogens is that the ligand-bound ER may have different transactivation properties when bound to different types of DNA enhancer elements. The classical estrogen response element (ERE) is composed of two inverted hexanucleotide repeats, and ligand-bound ER binds to the ERE as a homodimer (Fig. 1B). The ER also mediates gene transcription from an AP1 enhancer element that requires ligand and the AP1 transcription factors Fos and Jun for transcriptional activation (Fig. 1B) (*8*). In transactivation experiments, tamoxifen inhibits the transcription of genes that are regulated by a classical ERE, but like the natural estrogen hormone 17β-estradiol [E_2 (Fig. 1A)], tamoxifen activates the transcription of genes that are under the control of an AP1 element (*9*).

At the end of 1995, a second ER (ERβ) was cloned from a rat prostate cDNA library (*10*), and, subsequently, the human (*11*) and mouse (*12*) homologs were cloned. The first identified ER has been renamed ERα (*10*). The existence of two ERs presents another potential source of tissue-specific estrogen regulation. Here we compared the transactivation properties of ERα and ERβ with a panel of five ER ligands with the use of a reporter gene under the control of either a classical ERE or an AP1 element (*13*). Our results show that ERα and ERβ respond differently to certain ligands at an AP1 element. These results suggest different regulatory functions for the two ER subtypes.

We examined the transactivation properties of ERα (*14*) and ERβ (*15*) at a classical ERE in response to the estrogens E_2 and diethylstilbestrol (DES) and the antiestrogens Imperial Chemical Industries (ICI) 164384, tamoxifen, and raloxifene (*16*). We conducted these experiments by transfecting HeLa cells with either an ERα or ERβ expression plasmid along with a reporter plasmid that contained a luciferase gene under the transcriptional control of an ERE (*17*). Both ERα (*18*) and ERβ (Fig. 2) showed the same transactivation profiles with the panel of ligands. E_2 and DES stimulated luciferase production 10-fold over ICI 164384, raloxifene, tamoxifen, and the control (no ligand added). The antiestrogens blocked E_2 stimulation in ligand competition experiments (*18*).

We next examined the ligand-induced transactivation behavior of ERα and ERβ at an AP1 site. With ERα, all five ligands stimulated luciferase transcription, including the antiestrogens ICI 164384, tamoxifen, and raloxifene (Fig. 3). This stimulation was dependent on transfected ER, as cells trans-

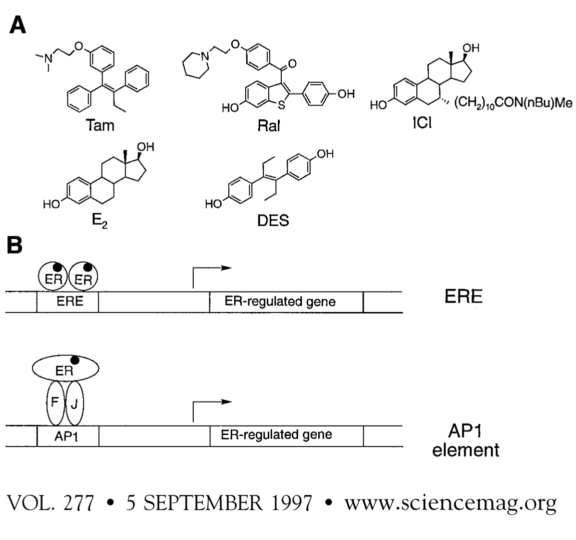

Fig. 1. (**A**) Structures of ER ligands. The estrogens E_2 and DES and the antiestrogens tamoxifen (Tam), raloxifene (Ral), and ICI 164384 (ICI) are shown. Bu, butyl; Me, methyl. (**B**) Models of ER action at a classical ERE and an ER-dependent AP1 response element. The filled circles represent the ligand bound to the ER. The AP1 proteins Jun and Fos are labeled J and F, respectively.

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fected with only the reporter plasmid showed no induction of reporter transcription (*18*). Of the five ligands, raloxifene induced transcription the least, showing twofold induction compared with the sixfold inductions typically seen with E_2 and tamoxifen. The raloxifene-induced transactivation was dose-dependent with a concentration value required for one-half maximal activation (EC_{50}) of about 1 nM (*18*). In addition, raloxifene reduced the activation caused by E_2 in a dose-dependent manner to the amount observed with raloxifene alone (*18*), demonstrating that raloxifene induction is weaker than induction by E_2 and that raloxifene-induced transactivation results from binding to ERα. If E_2 is classified as a full activator of ERα at an AP1 element (ERα-AP1), then raloxifene functions as a partial activator and tamoxifen functions as a full activator.

In contrast to the results seen with ERα-AP1, we observed a difference in the ligand activation profile of ERβ at an AP1 element (ERβ-AP1). In cells transfected with ERβ, treatment with the estrogens E_2 and DES did not increase luciferase transcription over the control (no ligand added), whereas treatment with the antiestrogens ICI 164384, raloxifene, and tamoxifen increased luciferase transcription (Fig. 4A). This transcription activation required transfected ERβ, as cells that were transfected with only the reporter plasmid did not show transcriptional activation by the antiestrogens (*18*). The transcriptional activation caused by raloxifene was dose-dependent with an EC_{50} value of about 50 nM (Fig. 4B). In ligand competition experiments, both E_2 and DES were able to block the raloxifene induction, and both estrogen ligands were able to reduce raloxifene induction to the basal level of transcription in a dose-dependent manner with concentration values required for one-half maximal inhibition of 1 to 10 nM (Fig. 4C). In a different ligand competition experiment, the inhibitory effect on transcription resulting from E_2 treatment could be overcome by higher concentrations of raloxifene in a dose-dependent manner (Fig. 4D). Thus, it appears that the pharmacology of ER ligands is reversed at an AP1 element with ERβ: with ERβ-AP1, the antiestrogens act as transcription activators, and the estrogens act as transcription inhibitors.

We next asked whether the action of ERβ-AP1 could be observed in cell lines derived from estrogen target tissues such as the uterus and breast. We performed transactivation assays for ERβ-AP1 in Ishikawa cells (a human uterine cell line) (Fig. 5A) and in MCF7 (Fig.

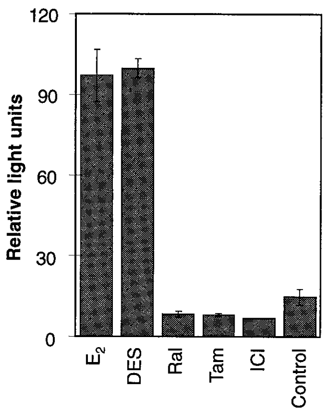

Fig. 2. ERβ action at an ERE. HeLa cells were transfected with an ERE-regulated luciferase reporter plasmid and an expression vector for rat ERβ (*15*). Transfected cells were treated with the five ligands (E_2, 0.1 μM; DES, 1 μM; Ral, 1 μM; Tam, 5 μM; and ICI, 1 μM) or an ethyl alcohol (EtOH) vehicle (control) (*17*). Error bars show deviations between wells from a single representative transfection.

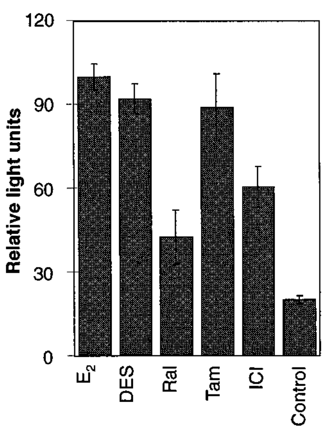

Fig. 3. ERα action at an AP1 element. HeLa cells were transfected with an AP1 reporter plasmid and an ERα (*14*) expression plasmid and treated with the five ligands (*17*). Ligand concentrations were E_2, 0.1 μM; DES, 1 μM; Ral, 1 μM; Tam, 5 μM; and ICI, 1 μM.

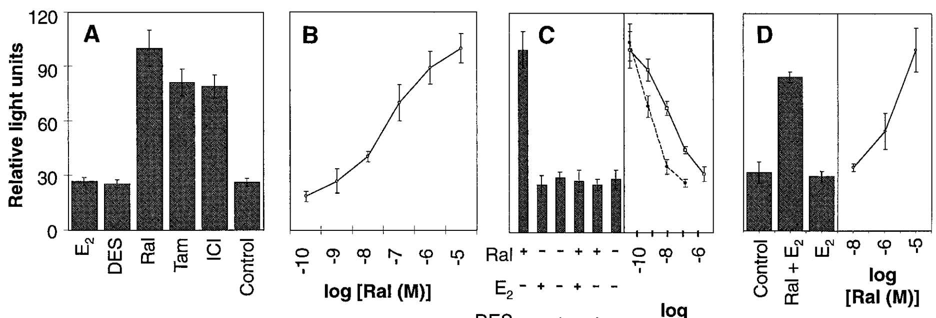

Fig. 4. (**A**) ERβ action at an AP1 response element. HeLa cells were transfected with an AP1 reporter plasmid and a rat ERβ expression plasmid (*15*). Transfected cells were treated with the following ligand concentrations: E_2, 0.1 μM; DES, 1 μM; Ral, 1 μM; Tam, 5 μM; and ICI, 1 μM (*17*). (**B**) Dose response of raloxifene induction with ERβ at an AP1 element. HeLa cells transfected as described for (A) were treated with the indicated range of raloxifene concentrations. (**C**) Competitive inhibition of raloxifene induction by E_2 and DES. HeLa cells were transfected as described for (A) and treated with ligands. The left panel shows transactivation induction by raloxifene (1 μM), the lack of induction by E_2 (0.1 μM) and DES (1 μM), and the ability of E_2 (0.1 μM) and DES (1 μM) to inhibit competitively raloxifene (1 μM) induction to the amount observed with the control (no ligand added). The right panel shows the dose dependence of inhibition of raloxifene (1 μM) induction by DES (solid line) and E_2 (dashed line). (**D**) Raloxifene overriding E_2 inhibition. HeLa cells were transfected as described for (A) and treated with ligands. The left panel shows the transcription induction resulting from the vehicle control (EtOH), Ral (10 μM) plus E_2 (10 nM), and E_2 (10 nM) alone. The right panel shows the dose dependence of raloxifene induction in the presence of E_2 (10 nM).

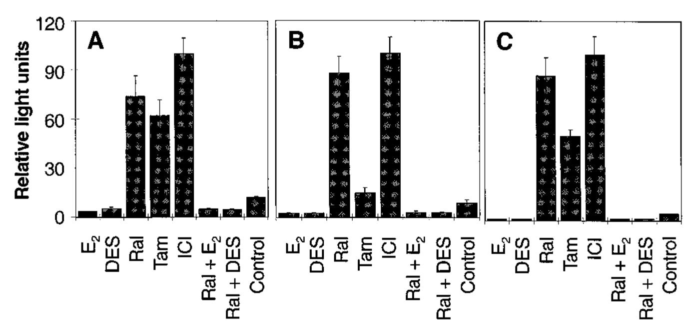

Fig. 5. (**A**) Ligand-dependent ERβ action at an AP1 element in Ishikawa cells. Ishikawa cells were transfected with an AP1-regulated luciferase reporter plasmid and an ERβ expression plasmid (*19*). Transfected cells were treated with one or two ligands as indicated (E_2, 0.1 μM; DES, 1 μM; Ral, 1 μM; Tam, 5 μM; and ICI, 1 μM) or an EtOH vehicle (control) (*17*). (**B**) Ligand-dependent ERβ action at an AP1 element in MCF7 cells. MCF7 cells were treated and analyzed as described for (A). (**C**) Ligand-dependent ERβ action at an AP1 element in MDA453 cells. MDA453 cells were treated and analyzed as described for (A).

5B) and MDA453 (Fig. 5C) human breast cancer cells (*19*). In each of these cell lines, the ligands acted the same as they did in the HeLa cells; the three antiestrogens activated and the estrogens inhibited ERβ-dependent transcription from an AP1 site (Fig. 5). No induction was seen with cells that were not transfected with the ERβ expression plasmid, indicating that the antiestrogen induction required ERβ (*18*). Antiestrogen induction in the breast cell lines was higher than that observed in HeLa cells. Transfected MCF7 cells treated with raloxifene gave a 20- to 80-fold transactivation response over the control (no ligand added). In addition, raloxifene and ICI 164384 induced transcription more than tamoxifen in the breast cell lines (Fig. 5, B and C). MCF7 cells do not appear to contain high concentrations of endogenous ERβ mRNA (*20*); however, our results suggest that the additional transactivation machinery required for ERβ-AP1 function is present in these cells. With two of these target tissue cell lines, E_2 treatment reduced the amount of transcription to less than that seen with the control (no ligand added). In MDA453 (Fig. 5C) and Ishikawa cells (Fig. 5A), E_2 treatment resulted in a consistent 40 to 75% reduction of reporter transcription levels compared with the control. This effect was also observed in ligand competition experiments (Fig. 5, A and C); E_2 and DES blocked raloxifene induction and reduced the amount of transcription to less than that seen for the control. Thus, when ERβ is bound by the estrogen hormone E_2 or the synthetic estrogen DES, it may function as a negative regulator of genes controlled by an ER-dependent AP1 element.

The ER is the only known member of the steroidal subfamily of nuclear receptors that has different subtypes (*21, 22*). Nuclear receptors that respond to nonsteroidal hormones that have different known subtypes include the thyroid receptor (TRα and TRβ), the retinoic acid receptor (RARα, RARβ, and RARγ), and the retinoid X receptor (RXRα, RXRβ, and RXRγ) (*23*). Our results demonstrate that two nuclear receptor subtypes can respond in opposite regulatory modes to the natural hormone from the same DNA response element. Moreover, the ligand-induced responses with ERβ at an AP1 site provide an example of negative transcriptional regulation by the natural hormone and strong positive regulation by synthetic antiestrogens (*24*).

If signaling from ER-dependent AP1 elements occurs in estrogen target tissues, our finding that ERα and ERβ respond differently to ligands at AP1 sites reveals a potential control mechanism for transcriptional regulation of estrogen-responsive genes and adds a layer of complexity in analyzing the pharmacology of antiestrogen therapeutics. The role of E_2 complexed to ERβ would be to turn off the transcription of these genes, whereas the antiestrogens raloxifene, tamoxifen, and ICI 164384 could overide this blockade and activate gene transcription. Thus, it may be helpful to search for genes in estrogen target tissues that are transcriptionally regulated by ERβ at an AP1 site and to characterize the phenotype of cells in which these genes are activated.

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13. The ERE- and AP1-driven luciferase reporter plasmids [(EREII-Luc GL450) and Δcoll73, respectively] and the ERα expression plasmid (pSG5-HEO) were used as previously described (*9*). The rat ERβ expression vector has been previously described (*10*). The full-length human ERβ cDNA, which was isolated from an ovarian cDNA library and found to be identical to the previously reported partial cDNA clone (*11*), was cloned into the pCMV5 eukaryotic expression vector (E. Enmark *et al.*, unpublished data), and the resulting ERβ expression vector was used for these experiments. Western blotting of rat mammary gland and prostate nuclear extracts probed with polyclonal antibodies raised against ERβ ligand-binding domain (LBD) and preadsorbed on a column of ERα LBD-coupled Sepharose showed that in both breast and prostate nuclear extracts the major band recognized by the antibody has the same mobility as full-length bacculovirus-expressed ERβ. This indicates that our ERβ expression vector encodes the major isoform of ERβ in these tissues (T. Rylander, M. P. Huikko, J.-Å. Gustafsson, unpublished data).
14. The data presented in this paper were obtained with the HEO ERα variant. HEO shows reduced transactivation response from the unliganded receptor compared with the wild-type ERα, resulting in clearer ligand-induced transactivation data. Each experiment with ERα was also checked with the wild-type ERα (HEGO), and the general ligand induction trends were found to be the same as those obtained with HEO. The only difference was that the ligand-induced transactivation responses were lower with HEGO than with the control (no ligand added).
15. Transactivation experiments were performed with both rat and human ERβ, and identical trends in ligand behavior were seen with both ERβs in HeLa cells.
16. Raloxifene was synthesized according to the published procedure (*3*). Structure and purity were verified by ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, ultraviolet spectroscopy, thin-layer chromatography, and high-resolution mass spectrometry.
17. Cells were grown in Nunc (Roskilde, Denmark) Delta Surface tissue culture plates to a density of not more than 2×10^5/cm^2. Cells were grown in sterile filtered Dulbecco's modified Eagle's–F-12 Coon's Modified Medium (Sigma Cell Culture) with 15 mM Hepes, L-glutamine (0.438 g/liter), $NaHCO_3$ (1.338 g/liter), 10% Seru-Max 4 (an iron-supplemented, formula-fed newborn calf serum, from a lot tested for low estrogenic activity; Sigma Cell Culture); gentamycin (0.05 mg/ml), streptomycin SO_4 (100 mg/ml), and penicillin "G" (100 U/ml). Ishikawa cells were grown in a medium containing 100 nM tamoxifen, and MCF7 cells were grown in a medium containing 10 nM estradiol. For the transfection assays, cells were suspended in 0.5 ml of electroporation buffer in 0.4-cm gap electroporation cuvettes (Bio-Rad) at 10^6 to 2×10^6 cells per cuvette. The electroporation buffer was prepared as a solution of 500 ml of phosphate-buffered saline (PBS), 5 ml of 10% glucose, and 50 μl of Biobrene. Five micrograms of reporter plasmid and 5 μg of ER expression plasmid were added, and the cuvette was agitated to facilitate mixing of the solution and homogeneous cell distribution in the cuvette. Cells were then immediately transfected by electroporation with a Bio-Rad GenePulser electroporation apparatus at an electric potential of 0.25 kV and a capacitance of 960 μF. We added 1 ml of growth medium (described above) to the electroporation cuvettes. The transfected cells for one experiment were pooled and carefully resuspended in the growth medium at a density of 8×10^4 to 1.6×10^5 cells/ml. After a homogeneous cell distribution was obtained by thorough mixing, cells were plated on Nunc six-well dishes at 2 ml per well. After 2 hours of incubation at 37°C, hormones were added, and the medium was mixed by gentle swirling. Cells were then incubated in the presence of hormone for 40 to 48 hours. The growth medium was removed from the wells, the cells were washed with Mg^{2+}- and Ca^{2+}-free PBS, and then they were lysed chemically [with 0.2 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 0.2% Triton X-100 and 1 mM dithiothreitol (DTT)]. The plates were then frozen to −80°C, thawed, and scraped with a rubber policeman to loosen and break up cell fragments. The lysate was centrifuged in a microfuge for 2 min, 0.1 ml of the supernatant was combined with 0.3 ml of the luciferase assay solution, and the chemiluminescence was measured immediately for a period of 10 s. The luciferase assay solution consists of 25 nM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, and 15 mM potassium phosphate at pH 7.8, with the addition of DTT to a final concentration of 1 mM, adenosine triphosphate to a final concentration of 2 mM, and luciferin (Analytical Luminescence Laboratories) to a final concentration of 200 μM shortly before commencement of the assay. Luminescence measurements were performed on a Monolight 1500 (Analytical Luminescence Laboratories). The relative light units reported have been adjusted to a scale of 100 for uniformity.
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24. The genes for transforming growth factor (*7*) and quinone reductase [M. M. Montano and B. S. Katzenellenbogan, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2581 (1997)] are ER-regulated genes controlled by promoters containing nonclassical EREs that are activated by antiestrogens; however, the action of ERβ at either of these promoters has not been reported. The action of ERα on the quinone reductase gene shows a similar ligand profile to that of ERβ at an AP1 site; antiestrogens are transcription activators, and E_2 is a transcription inhibitor.

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