Mouse Estrogen Receptor β Isoforms Exhibit Differences in Ligand Selectivity and Coactivator Recruitment

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ABSTRACT: Estrogens exert their physiological effects through two estrogen receptor (ER) subtypes, ERα and ERβ. In mouse, the cloning of an alternative splice variant of the wild-type ERβ (mERβ1), mERβ2, which contains an 18 amino acid insertion in the ligand binding domain, contributed an additional level of complexity to estrogen signaling. In this study we have assayed the interaction of several known ligands with mERβ1 and mERβ2. The binding affinity of estradiol was 14-fold higher for mERβ1 than for mERβ2. In contrast, raloxifene was dramatically (8-fold) mERβ2 selective. The selectivity for mERβ2 was abolished when the 2-arylbenzothiophene core of the raloxifene molecule was tested for binding affinity, demonstrating that the 3-aroyl side chain of raloxifene plays an important role in contributing to its mERβ2 selectivity. The opposite isoform selectivity found for estradiol and raloxifene in our ligand binding assay was also reflected in the transactivation assay system. That is, mERβ2 required 10-fold greater estradiol concentrations for maximal activation compared to mERβ1, whereas raloxifene was more potent in antagonizing estradiol-induced gene expression via mERβ2 than mERβ1. The raloxifene core behaved as a pure agonist. Furthermore, mERβ2 showed significantly decreased estradiol-induced maximal transcriptional activity as compared to mERβ1. A pull-down assay indicated that the interactions of TIF2 and RAP250 with mERβ2 were weaker than with mERβ1. To assess TIF2 and RAP250 interactions with ERs more quantitatively, we examined the interaction of LXXLL containing peptides derived from TIF2 and RAP250 with mERβ1 and mERβ2 using surface plasmon resonance analysis. Our results indicate that mERβ2 interacts with both coactivators with lower affinity, which may explain its reduced transcriptional activity. Taken together, these results suggest that ligand selectivity and coactivator recruitment of the ERβ isoforms constitute additional levels of specificity that influence the transcriptional response in estrogen target cells.

Estrogens exert their physiological effects through two estrogen receptor (ER)[1] subtypes, ERα and ERβ, that belong to a large family of transcription factors, the nuclear receptor family (*1*). ERα and ERβ contain characteristic sequence motifs associated with transactivation, DNA binding, and hormone binding (*2*). They share high homology within the DNA binding domain and modest homology within the ligand binding domain (LBD). The LBD is multifunctional and, in addition to harboring the ligand binding pocket, encompasses regions for receptor dimerization and ligand-dependent (AF-2) transactivation (*3*). Hormone binding to the ER LBD induces a conformational change in the receptor that initiates a series of events that culminate in the activation or repression of responsive genes (*4*). Although the precise mechanism by which ER affects gene transcription remains to be determined, it appears to be mediated, at least in the case of AF-2 activation, via nuclear receptor coregulators that are recruited by the DNA-bound receptor (*5*).

To date, a number of ERβ mRNA isoforms, generated by alternative mRNA splicing, have been described in human, mouse, and rat. In mouse, the originally described wild-type form has been named mERβ1 (*6*). mERβ2 is an alternative splice variant where an additional exon is spliced in-frame between exons 5 and 6 to generate a protein with an 18 amino acid insertion in the LBD (*7*). The inserted amino acid sequence shares significant (16 out of 18 amino acids) homology with the respective insert in the rat ERβ2 (rERβ2) isoform (*8*). It was found that mERβ1 and mERβ2 are expressed at similar relative levels in some tissues such as the ovary and lung. However, in a range of tissues such as liver, pancreas, gut, and bone, mERβ2 mRNA is more abundant (*7*, *8*). In ligand binding assays ERβ2 binds estradiol with a lower affinity than ERβ1 (*9*−*12*). In a reporter assay, ERβ2 shows lower transcriptional activity than ERβ1 and acts as a negative regulator when it is coexpressed with ERα or ERβ1 (*12*). The physiological role of rodent ERβ2 remains to be determined. It was shown that rERβ2 protein was upregulated during the lactation period, suggesting that rERβ2 may play a role in silencing of ERα function during lactation in rat mammary gland (*13*).

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[1] Abbreviations: ER, estrogen receptor; LBD, ligand binding domain; SPR, surface plasmon resonance.

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Ligand-dependent interaction of nuclear receptors and coactivators is a critical step in nuclear receptor-mediated transcriptional regulation. The most studied coactivators for nuclear receptors belong to the steroid receptor coactivator 1 (SRC-1) family, which contains three related members, referred to as SRC-1, SRC-2/GRIP1/TIF2, and SRC-3/p/CIP/RAC3/ACTR/AIB1/TRAM1 (*14*). Critical for the function of these coactivators is the central nuclear receptor interaction domain consisting of three equally spaced conserved LXXLL motifs, also called nuclear receptor boxes. Nuclear receptor-activating protein 250 (RAP250), also called ASC-2, PRIP, TRBP, and NRC, was recently cloned and described as a novel nuclear receptor coactivator consisting of two LXXLL motifs, of which only the N-terminal motif interacts with nuclear receptors in general (*15*).

In this paper we address the characteristics of two mouse ERβ isoforms, mERβ1 and mERβ2, with regard to ligand selectivity and transcriptional activation.

EXPERIMENTAL PROCEDURES

Materials. 17β-Estradiol, 4-OH-tamoxifen, genistein, and raloxifene were from Sigma-Aldrich Sweden AB. ICI-182,-780 was obtained from Tocris, Inc. The raloxifene core [2-(4-hydroxyphenyl)benzo[*b*]thiophen-6-ol] was prepared at Karo Bio AB (Sweden) according to the method of Jones et al. (*16*). The radioligand [^{3}H]-17β-estradiol was purchased from PerkinElmer Life Sciences Inc.

Plasmids, Transient Transfection Assays, and Western Blot Analysis. The pSG5-mERβ1 expression plasmid was a gift from Dr. K. Pettersson at the Department of Biosciences, Karolinska Institutet. This expression vector includes the cDNA that encodes the full-length murine ERβ (549 aa) (*17*). The murine ERβ2 expression plasmid (pSG5-mERβ2) was constructed by replacement of the murine ERβ1 cDNA sequences spanning nucleotides 841−1200 (GenBank, U81451) with the corresponding region of the murine ERβ2 generated by RT-PCR, using *Sac*I/*Bst*BI restriction sites. The sequences of the plasmids were verified by DNA sequencing.

HEK293 cells were cultured in a 1:1 mixture of Ham's nutrient mixture F12 (Invitrogen) and DMEM (Invitrogen) supplemented with 5% FBS, 100 units of penicillin/mL, and 100 μg of streptomycin/mL. For transfection, cells were seeded at a density of 5×10^4 cells/well in 24-well plates and cotransfected with 2 × ERE TK luciferase reporter plasmid (0.8 μg) together with pSG5-mERβ1 or pSG5-mERβ2 (0.016 μg) expression plasmids. A pRL-TK control plasmid, which contains a *Renilla* luciferase gene, was included to control for differences in transfection efficiencies. Transfections using the Superfect reagent (Qiagen) were performed according to the manufacturer's protocol. The medium was replaced with a phenol red-free mixture of F12 and DMEM containing 5% dextran-coated charcoal-treated FBS, 100 units of penicillin/mL, and 100 μg of streptomycin/mL upon transfection. Estradiol, raloxifene, the raloxifene core, or vehicle (in 0.1% ethanol) was added immediately after transfection. The cells were harvested 24 h after transfection, and luciferase activities were determined using the dual luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Western blotting was done according to the protocol as described (*13*). ERβ was detected with an ERβ LBD rabbit polyclonal antibody produced by us as described previously (*18*).

Cloning and Expression of mERβ1 and mERβ2 Ligand Binding Domains. The LBDs of murine ERβ1 (R209 to Q485, GenBank, U81451) and the corresponding region of murine ERβ2, containing the 18 aa insertion, were generated by PCR using pSG5-mERβ1 and pSG5-mERβ2 as templates, respectively, and primers that contained appropriate restriction sites. The LBDs of mERβ1 and mERβ2 were cloned into pET15b (Novagen, Madison, WI) to generate proteins with N-terminal His tags. The sequences of the constructs were verified by DNA sequencing.

Cultures (500 mL) of the *Escherichia coli* strain BL21, transformed with the appropriate expression plasmids, were cultivated overnight in LB supplemented with 100 μg/mL ampicillin at 37 °C. When the OD_{600} reached 1.0, IPTG was added to a final concentration of 1 mM and incubation continued for 3 h at 25 °C. The cells were pelleted, and the supernatant was discarded. The pellet was suspended in 5 mL of extraction buffer (0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 0.01 mg/mL DNase, 0.01 mg/mL RNase, 10 mM $MgCl_2$, 0.25 mg/mL lysozyme, 1 mM β-mercaptoethanol, and protease inhibitor cocktail tablet). The samples were sonicated for 4 min at 50% duty (total sonication time 2 min). The homogenate was centrifuged at 20000*g* for 20 min at 4 °C. The supernatant was applied to a TALON metal affinity column (Clontech Laboratories, Inc., Palo Alto, CA). Fractions containing the purified protein were dialyzed against 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM DTT and frozen at −80 °C. The purified protein was more than 95% pure as determined from Coomassie-stained SDS−PAGE gels. The protein concentrations were measured using the Coomassie protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Scintillation Proximity Assay (SPA). The assay was performed in 96-well microplates (PerkinElmer Life Sciences Inc., Boston, MA). Polyvinyltoluene (PVT) copper-loaded His-tag beads were purchased from Amersham Corp. The reaction mixture (60 μL per well) containing assay buffer (1 mM EDTA, 0.9 M KH_2PO_4, 0.1 M K_2HPO_4, 20 mM Na_2-MoO_4, and 0.05% monothioglycerol), beads (30 μg/well), and purified ERβ LBD (final concentration of 5 nM) was incubated at 4 °C for at least 1 h. The concentration of active receptors that are able to bind ligand was 0.6 nM, calculated from B_{max} (the maximal density of receptor sites). For saturation ligand binding analysis, a sample of various concentrations of [^{3}H]-17β-estradiol (SA = 95 Ci/mmol) in the presence or absence of a 300-fold excess of unlabeled 17β-estradiol was then added. The assay plates were sealed, allowed to settle overnight, and subsequently counted on a Wallac 1450 micro-β-counter. The dissociation constant (K_d) was calculated as the free concentration of radioligand at half-maximal specific binding by fitting data to the Hill equation and by linear Scatchard transformation (*19*). Curve fitting was done in Prism (GraphPad Software Inc.).

For ligand competition studies, purified ERβ LBDs (5 nM) were incubated overnight at 4 °C with a range of test compound concentrations. A final concentration of 1.5 nM [^{3}H]-17β-estradiol (30 μL per well) was used. The ligands were tested three times with similar results. Curve fitting was performed using Prism (GraphPad Software Inc.), and the IC_{50}s were determined. IC_{50} values were converted to K_i

using the Cheng−Prusoff equation, $K_i = IC_{50}/(1 + D/K_d)$, where D is the concentration of the radioligand (*20*).

Surface Plasmon Resonance (SPR) Analysis for Raloxifene Binding. SPR analyses were performed according to the protocol as described (*21*). Research grade CM5 sensor chips were obtained from BIAcore AB. Penta-His antibody (Qiagen) surfaces were prepared using standard amine coupling procedures.

Plasmids and Pull-Down Assay. TIF2 and RAP250 for in vitro translation were expressed from the previously described plasmids, pSG5Gal4-RAP250 (aa 819−1096) (*15*) containing the first LXXLL motif and pBKCMV-TIF2 (*22*) containing full-length TIF2, respectively.

For pull-down assays, purified His-tagged ERβ LBDs (100 μg) were bound to 60 μL of Talon resin and then equilibrated in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, and 0.5% NP-40 (equilibration buffer). The gel slurry was then divided into two equal aliquots, and to each tube was added 2.5 μL of in vitro translated, ^{35}S-labeled (TNT coupled reticulocyte lysate system, Promega), TIF2 or RAP250 in a total volume of 150 μL of equilibration buffer containing 1.5% BSA. Estradiol or vehicle (ethanol) was added as indicated. As control, TIF2 or RAP250 was mixed with Talon gel without bound ERβ. All samples were incubated for 2 h with gentle shaking at 4 °C. After washing three times with equilibration buffer, bound proteins were eluted with SDS−PAGE sample buffer and separated on a 12% polyacrylamide gel. The gel was stained with Coomassie blue, followed by determination of ^{35}S using a Phosphoimager instrument.

Surface Plasmon Resonance (SPR) Analysis for Coactivator Binding. All SPR measurements were performed on a BIAcore 2000 instrument (BIAcore AB, Uppsala, Sweden). All experiments were performed at 25 °C and at a flow rate of 5 μL/min. Research grade streptavidin sensor chips were obtained from BIAcore AB. The streptavidin chips were first treated with three 1-min pulses of 50 mM NaOH and 1 M NaCl at a flow rate of 5 μL/min. N-Terminally biotinylated peptides (>95% purity) were purchased from Interactiva (Germany). LXXLL peptide sequences were as follows: TIF2 Box1 (residues 636−649), KGQTKLLQLLTTKS; TIF2 Box2 (residues 685−698), EKHKILHRLLQDSS; TIF2 Box3 (residues 742−755), KENALLRYLLDKDD; RAP250 Box1 (residues 882−895), LTSPLLVNLLQSDI. Peptides were immobilized on individual surfaces to 200 RU responses, and a non-LXXLL peptide was immobilized on a control surface for on-line reference subtraction. Samples of purified mERβ1 LBD or mERβ2 LBD were then injected over each surface. After injection stopped, the surfaces were washed with buffer to monitor the dissociation phase. The buffer used was 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.05% Tween 20. For the kinetic measurements, various concentrations of mERβ1 LBD or mERβ2 LBD (from 20 to 100 nM) were injected over the chip surfaces. The BIAevaluation software version 3.1 was used for evaluation. Different binding models (different rate equations) were tested in the global curve fitting procedure, and the model best describing the experimental data was a conformational change model. The apparent K_D values are calculated as described (*23*).

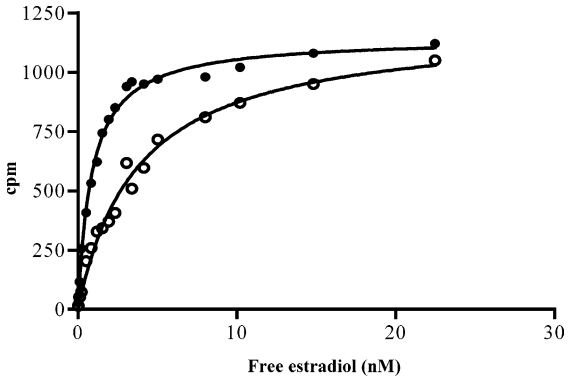


FIGURE 1: Saturation analyses for [^{3}H]-17β-estradiol binding to mERβ1 and mERβ2. mERβ1 (●) and mERβ2 (○) LBDs were incubated with increasing concentrations (0−30 nM) of [^{3}H]-17β-estradiol at 4 °C overnight. Nonlinear regression in Prism (GraphPad Software) of the data gives a K_d of 0.8 nM for mERβ1 and a K_d of 4.2 nM for mERβ2.

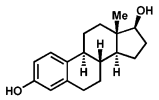
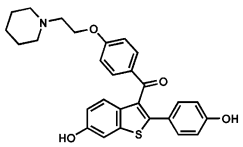
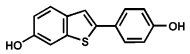
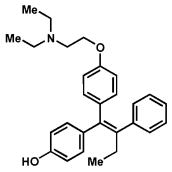
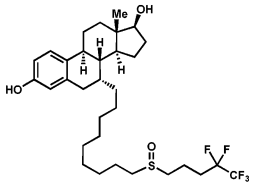
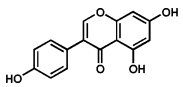
RESULTS

mERβ1 and mERβ2 Display Isoform Selectivity with Regard to Ligand Binding. The C-terminal regions containing the LBD of mERβ1 and mERβ2 were expressed in *E. coli*, and the recombinant proteins were purified. Ligand binding to purified proteins was measured using a scintillation proximity assay. In Figure 1, the results of saturation ligand binding assays with tritiated estradiol are shown. The measured K_d values were 0.8 nM for mERβ1 and 5-fold higher (K_d = 4.2 nM) for mERβ2. We next compared the binding affinities of mERβ1 and mERβ2 to a set of ER ligands using a competition assay. Table 1 shows the K_is of these compounds. As expected, the selectivity of estradiol was higher for mERβ1 than for mERβ2 (14-fold). Interestingly, raloxifene was dramatically (8-fold) mERβ2 selective. When the raloxifene core was tested in the ligand binding assays, the selectivity for mERβ2 was abolished, confirming that the selectivity for mERβ2 was due to, at least in part, the 3-aroyl side chain of raloxifene. No striking differences in the binding affinities for 4-OH-tamoxifen, ICI-182,780, or genistein between mERβ1 and mERβ2 could be detected. These data indicate that ER ligands exhibit distinct selectivity for mouse ERβ isoforms.

To further confirm the higher binding affinity of mERβ2 compared to mERβ1 for raloxifene, we used SPR analysis to directly assay binding affinities. Figure 2 depicts the binding responses obtained for the concentration series of raloxifene injected across the mERβ1 or mERβ2 surface. It is clear from a visual inspection of the data that raloxifene dissociates from the mERβ1 faster than the mERβ2 isoform. The measured K_D values were 0.31 μM for mERβ1 and 10-fold lower (K_D = 0.03 μM) for mERβ2, confirming that raloxifene has a higher affinity for mERβ2 as compared to mERβ1.

mERβ1 and mERβ2 Isoform Selectivity Is Maintained in Transcriptional Regulation. Estradiol and raloxifene exhibited different isoform selectivity with regard to ligand binding for the mouse ERβ. Next, we examined how this selectivity was translated into modulation of transcriptional activity. Estradiol induced a concentration-dependent activation of mERβ1 and mERβ2 from a 2 × ERE driven reporter gene in transiently transfected HEK293 cells (Figure 3A). Notably, mERβ1 had higher estradiol-induced activity than mERβ2.

Table 1: Binding Affinity of Selected Ligands for mERβ1 and mERβ2

Compound	Structure	Ki (nM)	
		mERβ1	mERβ2
17-β-Estradiol		0.15	2.13
Raloxifene		23.31	3.02
Raloxifene core		3.42	20.40
4-OH-tamoxifen		1.50	3.03
ICI-182,780		56.20	45.92
Genistein		2.82	8.13

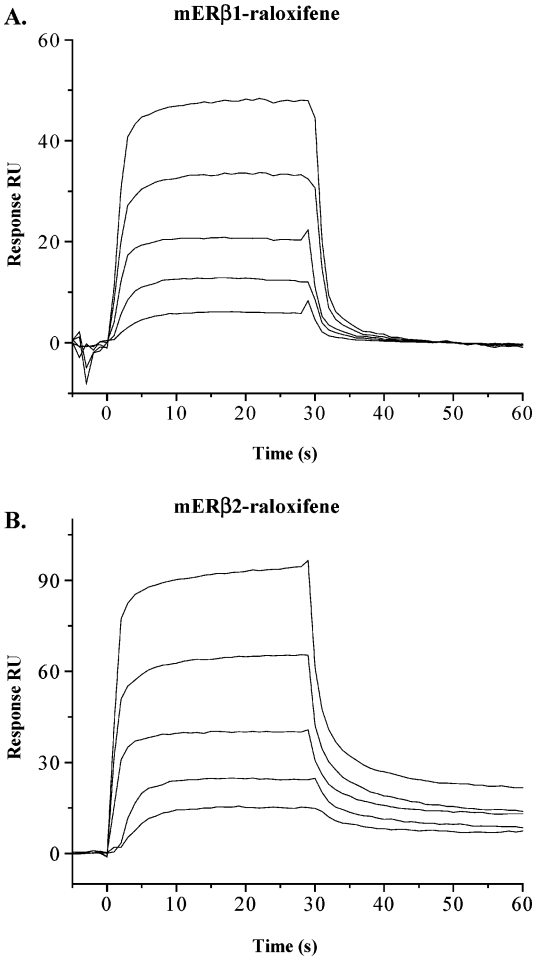


FIGURE 2: Kinetic analysis of raloxifene binding to mERβ1 and mERβ2. Raloxifene was injected at concentrations of 0.03125, 0.0625, 0.125, 0.25, and 0.5 μM over captured mERβ1 (A) and mERβ2 (B). The data were best described using a simple 1:1 interaction model, yielding a K_D of 0.31 μM for mERβ1 and a K_D of 0.03 μM for mERβ2.

Thus, maximal transcriptional activation by mERβ1 was observed at 1 nM estradiol, whereas maximal transactivation by mERβ2 was observed at 10 nM estradiol, at only 60% of the maximal level seen with mERβ1. Raloxifene showed only antagonist activity on both mERβ1 and mERβ2. As shown in Figure 3B, raloxifene was more potent in antagonizing estradiol-induced gene expression with mERβ2 than with mERβ1, which is in agreement with its higher relative binding affinity to mERβ2 than to mERβ1. In contrast to raloxifene, the raloxifene core behaved as a pure agonist for both mERβ1 and mERβ2 (Figure 3C). To confirm equivalent ERβ expression, extracts from HEK293 cells transfected with equal amounts of the expression vectors for mERβ1 or mERβ2 used in transient transfection assays were separated by SDS−PAGE and analyzed for ERβ expression by Western blot (Figure 3D). This analysis shows that mERβ1 and mERβ2 are expressed at similar levels.

mERβ2 Has Reduced Interaction with Coactivators. As shown in Figure 3A, mERβ2 displayed significantly decreased estradiol-induced maximal transcriptional activity as compared to mERβ1. Because the interaction of ER with coactivators is believed to determine the magnitude of transcriptional activity of the receptor, we compared the affinities of mERβ1 and mERβ2 to the coactivators TIF2 and RAP250. We performed pull-down assays using purified His-tagged ERβ LBDs and ^{35}S-labeled TIF2 or RAP250 in the presence or absence of estradiol. As shown in Figure 4, the binding of TIF2 and RAP250 to mERβ1 and mERβ2 appeared to be enhanced by the presence of estradiol. Furthermore, the interactions of TIF2 and RAP250 with mERβ2 were weaker than with mERβ1.

To examine interactions of TIF2 and RAP250 with ERs more quantitatively, we used SPR analysis, where biotinylated 14-mer peptides containing NR-box motifs (LXXLL) from TIF2 or RAP250 were captured via streptavidin to the chip surface. Figure 5A demonstrates overlaid sensorgrams of injections of unliganded mERβ2 or mERβ2 liganded with estradiol, 4-OH-tamoxifen, genistein, ICI-182,780, or raloxifene assayed for binding to the TIF2 NR-Box2 peptide. These results indicate that binding of ER agonists, estradiol and genistein, to mERβ2 enhances the mERβ2−peptide interaction, whereas 4-OH-tamoxifen, ICI-182,780, and raloxifene decrease the affinity. Similar differences in binding, depending on ligand status, were seen with mERβ1 and also with RAP250 interaction (data not shown). These data suggest that conformational changes, induced by ligand binding, impose different affinities for receptor−coactivator interactions. In Figure 5B, the concentration-dependent association of estradiol-bound mERβ2 to RAP250 NR-Box1 or TIF2 NR-Box2 is shown together with the best calculated fit. Similar binding studies between mERβ1 and all the different TIF2 and RAP250 NR-box peptides were also performed (data not shown). Using BIAevaluation software, the affinity of receptor−peptide interaction was determined. The best

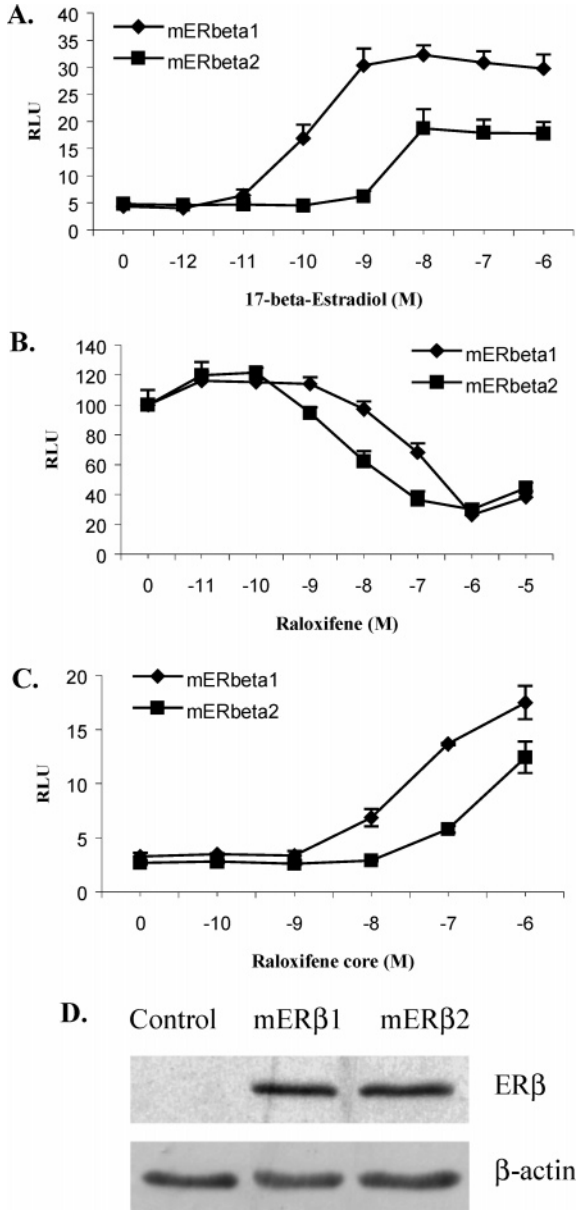


FIGURE 3: Transcriptional activity of mERβ1 and mERβ2 proteins assayed on 2 × ERE TK luciferase reporter. HEK293 cells were transfected with the 2 × ERE TK luciferase reporter plasmid and expression plasmids encoding mERβ1 or mERβ2. (A) Cells were treated with vehicle (ethanol) or the indicated concentrations of 17β-estradiol. (B) Antagonist potency of raloxifene for mERβ1 and mERβ2 in the presence of 10 nM 17β-estradiol. Cells were treated with vehicle (ethanol) or the indicated concentrations of raloxifene. Values obtained from cells treated with only 10 nM 17β-estradiol were arbitrarily set to 100. (C) Cells were treated with vehicle (ethanol) or the indicated concentrations of the raloxifene core. Values represent the mean ± SD of three independent experiments. (D) Western blot analysis of ERβ and β-actin. Identical amounts (100 μg of protein) of whole cell extracts from nontransfected control HEK293 cells or cells transfected with equal amounts of expression vectors for mERβ1 and mERβ2, respectively, were analyzed. The bands were quantified by densitometric scanning, and the amount of ERβ was normalized to β-actin.

fit for all peptide−ER interactions tested was obtained using a conformational change model. The apparent affinities listed in Table 2 show that the TIF2 peptides have higher affinity for mERβ1 and mERβ2 than the RAP250 peptide. The data also show that both TIF2 and RAP250 have binding preferences for mERβ1. In comparison to mERβ1, the lower affinity of mERβ2 to coactivators may account for its reduced maximal transcriptional activity.

DISCUSSION

Since 1997, when a new isoform of the rat ERβ gene named ERβ2 was identified (*8*), only a few studies have addressed the function of this variant (*9*−*12*). It has been shown that ERβ2 binds estradiol with a lower affinity than ERβ1. ERβ2 can form heterodimers with ERβ1 as well as with ERα. Further, transient coexpression of ERβ2 and ERα or ERβ1 in cell lines results in ERβ2-induced reduction of ERα and ERβ1 activity. In this paper, we have addressed the effect of ligand binding selectivity and ligand-induced recruitment of coactivators on the transcriptional activation profiles of mouse ERβ1 and ERβ2.

It is predicted that the 18 amino acid insertion of ERβ2 lies within helix 6 of the receptor (*24*). Given that the insertion is right after the β-sheet that contains Phe-356, which makes direct contact with the ligands, and in close proximity to helix 5, known to have direct interactions with coactivators, the inserted amino acids might affect both ligand binding and coactivator interactions. We have compared the binding affinities of mERβ1 and mERβ2 to a set of ER ligands. In accordance with previous studies, our results show that the binding affinity of estradiol for mERβ1 was much higher than for mERβ2. On the contrary, raloxifene was found to be dramatically mERβ2 selective. The differences found for estradiol and raloxifene in our ligand binding assay were also reflected in the transactivation assay. This observation suggests that raloxifene could act as a potent antagonist of ERβ2 actions and that this differential raloxifene sensitivity may represent a way to dissect the physiological importance of ERβ2. Furthermore, we demonstrated that the raloxifene core behaved as an agonist, showing that the selective antagonist potency of raloxifene for mERβ2 is due to its 3-aroyl side chain. Raloxifene but not 4-OH-tamoxifen or ICI has been shown to be a higher affinity ligand and a more potent estrogen antagonist for human ERα than ERβ (*25*). Therefore, the mERβ2 binding selectivity for these three ligands more closely resembles that of ERα than of mERβ1. In the present study, we also observed that mERβ2 showed significantly decreased estradiol-induced maximal transcriptional activity than mERβ1. We therefore evaluated the affinities of mERβ1 and mERβ2 for coactivators TIF2 and RAP250. Our results indicate that mERβ2 interacts with both coactivators with lower affinity than mERβ1, which may explain why the transcriptional activity of mERβ2 is lower than that of mERβ1.

A detailed understanding of why raloxifene is selective for mERβ2 over mERβ1 is not possible since no crystallographic structure of the ERβ2 isoform is currently available. However, a comparison of the existing raloxifene structures complexed with hERα (*26*) (PDB accession code 1ERR) and rERβ1 (*27*) (PDB accession code 1QKN) is instructive. As mentioned above, the raloxifene binding characteristics of mERβ2 more closely resemble ERα than ERβ1. As shown in Figure 6A, Phe-322 in the ERβ/raloxifene crystallographic structure is pointed toward the ligand forcing the 2-aryl group of raloxifene upward. As a consequence, the hydrogen bond between His-430 and the 4′-hydroxyl group of raloxifene is weakened (O···H−N angle

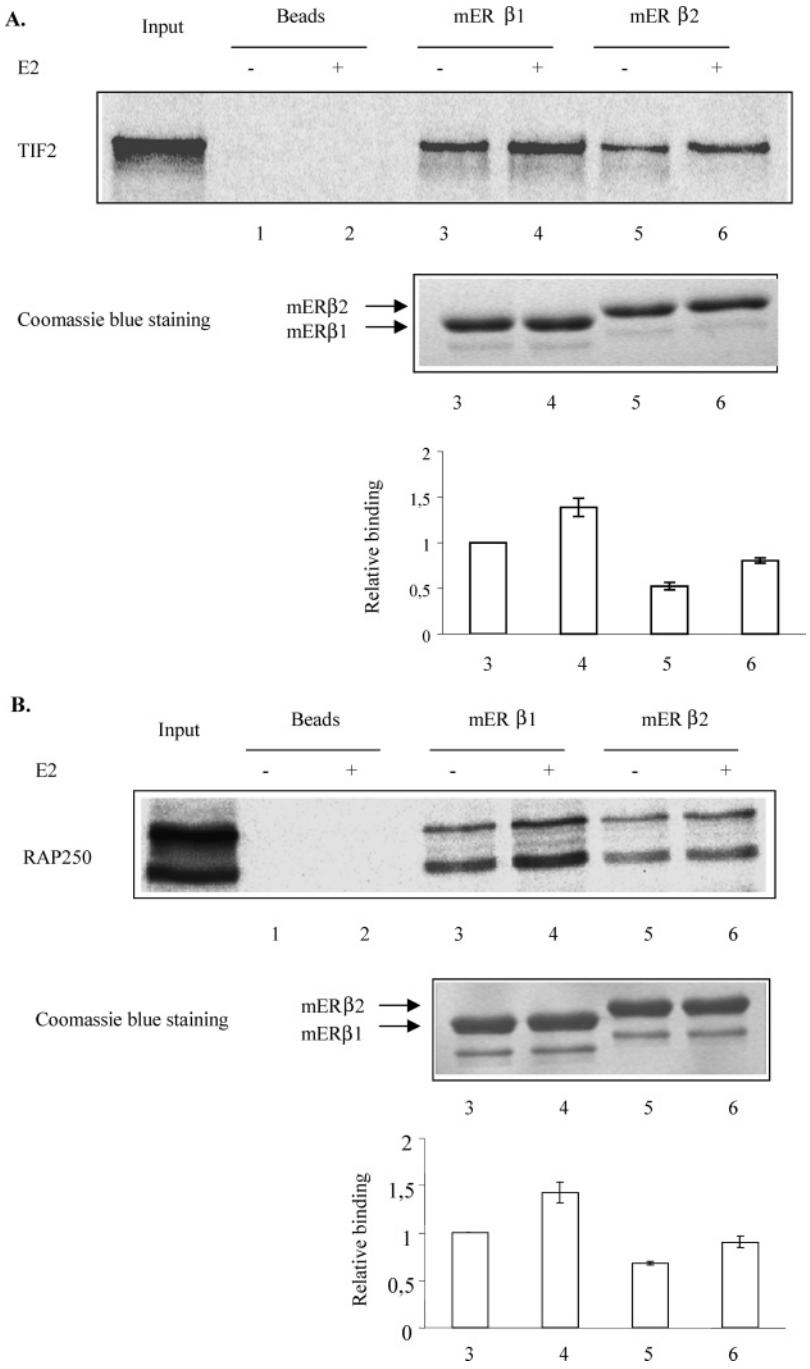


FIGURE 4: Analysis of TIF2 (A) and RAP250 (B) interactions with mERβ1 and mERβ2 by pull-down assay. In vitro translated TIF2 or RAP250 was incubated with mERβ1 (lanes 3 and 4) and mERβ2 (lanes 5 and 6), with or without estradiol (E2) as indicated. Nonspecific binding of TIF2 or RAP250 to Talon beads is shown in lanes 1 and 2. Input corresponds to 0.5 μL of the in vitro translation mixture. The amount of loaded mERβ1 (lanes 3 and 4) and mERβ2 (lanes 5 and 6) was examined by Coomassie blue staining (middle panel). The bar graph shows the relative binding of mERβ1 (lanes 3 and 4) and mERβ2 (lanes 5 and 6) to TIF2 or RAP250 compared to the amount of loaded protein, as determined by densitometric scanning. The relative binding of mERβ1 to TIF2 or RAP250 in the absence of estradiol was set to 1. Nonspecific binding of TIF2 or RAP250 to a 6 × His control protein, which does not interact with coactivators, could not be detected using the experimental conditions employed (data not shown).

= 145° vs 180° for an ideal hydrogen bond). In contrast, the corresponding Phe-425 is pointed away from the ligand in the ERα crystallographic structure, allowing more flexibility for positioning of the ligand in the binding cavity, and consequently the hydrogen bond between His-524 and the 4′-hydroxyl group is stronger (O···H−N angle = 165° in 1ERR). Hence the conformation of Phe-322 is an important determinant of the affinity of the various ER isoforms for raloxifene. Now turning to ERβ2, the insert between Arg-364 and Asp-365 on helix 6 is very close to Phe-322 (Figure 6A) and therefore could easily influence the preferred conformation of this phenylalanine residue. The ERβ2 insert may perturb the conformation of Phe-322 so that it behaves more like in ERα, making more room for the 2-aryl group of raloxifene so that in turn the 4′-hydroxyl group can form a stronger hydrogen bond to His-430. This would account for the increased affinity of raloxifene for ERβ2. The explanation for why raloxifene but not the raloxifene core is ERβ2 selective can be understood by comparing the crystallographic structure of ERα complexed

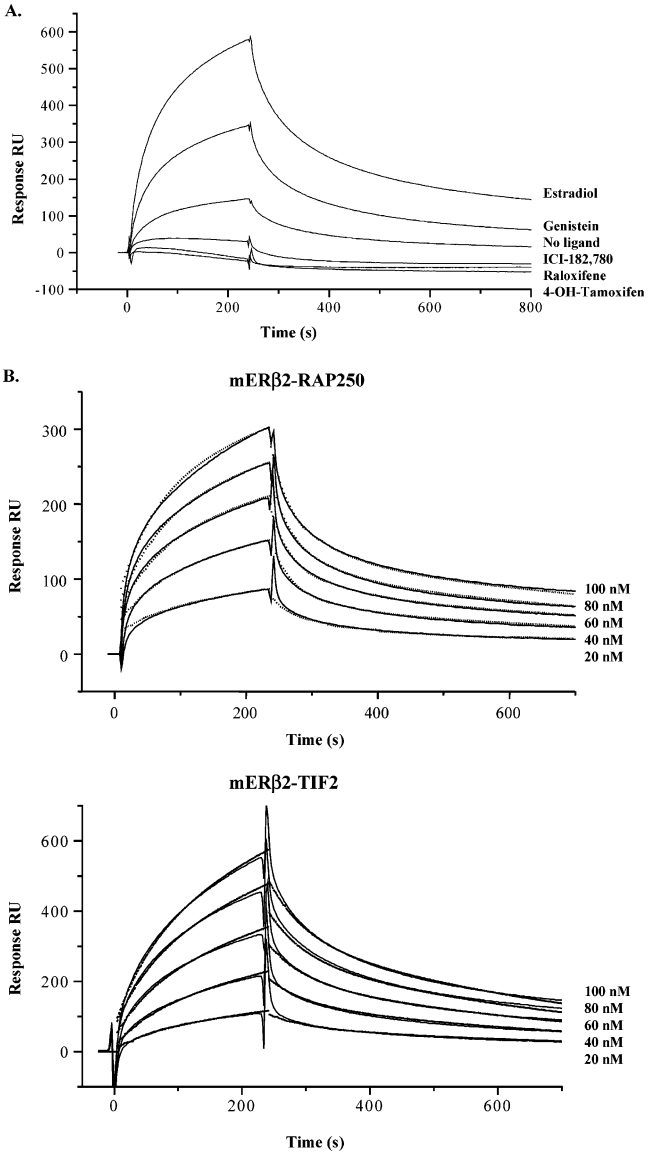


FIGURE 5: SPR analysis of LXXLL peptide binding to mERβ2. (A) Overlaid sensorgrams showing injections of 100 nM unliganded mERβ2 and mERβ2 liganded with estradiol, genistein, ICI-182,-780, raloxifene, or 4-OH-tamoxifen, respectively, over a surface captured with 200 RU of TIF2 NR-Box2 peptide. Ligand concentrations of 1 μM were used. (B) Overlaid sensorgrams showing injections of mERβ2 liganded with 1 μM estradiol at protein concentrations of 20, 40, 60, 80, and 100 nM over a surface captured with 200 RU of RAP250 NR-Box1 peptide or TIF2 NR-Box2 (solid lines) and best calculated fit using a conformational change model (dotted lines).

with the raloxifene core (PDB accession code 1GWQ) (*28*) to the two raloxifene structures, 1ERR and 1QKN (see Figure 6B). The raloxifene core binds to ER in a flipped orientation relative to raloxifene. In this binding mode, the 6-hydroxyl group of the raloxifene core forms a strong hydrogen bond to His-524 (O$\cdots$H$-$N angle $=$ 179°). Also, in this flipped orientation, the distance between the raloxifene core and Phe-425 is larger so that it is less sensitive to the conformation of this phenylalanine residue. In contrast, the 3-aroyl side chain forces the benzothiophene moiety of raloxifene to bind in a flipped orientation relative to the raloxifene core and pushes the 2-aryl group toward Phe-322.

The biological significance of the existence of two main ERβ isoforms in rat and mouse is presently unclear. Given

Table 2: Apparent Dissociation Constants and Rate Constants for Interactions between TIF2 or RAP250 NR-Box Peptides and Estradiol-Bound mERβ1 and mERβ2[a]

	TIF2			RAP250
mERβ1	Box1	Box2	Box3	Box1
K_D (nM)	7.3 ± 2.9	4.2 ± 0.8	12.7 ± 4.1	41.5 ± 6.9
$k_{a1} \times 10^4$ ($M^{-1}\,s^{-1}$)	12.1 ± 1.0	35.3 ± 2.3	15.1 ± 0.6	10.9 ± 0.3
$k_{d1} \times 10^{-3}$ (s^{-1})	3.4 ± 0.6	3.6 ± 0.8	3.3 ± 0.9	6.1 ± 0.1
$k_{a2} \times 10^{-3}$ (s^{-1})	1.9 ± 0.1	1.2 ± 0.2	1.8 ± 0.2	2.3 ± 0.1
$k_{d2} \times 10^{-3}$ (s^{-1})	0.5 ± 0.1	0.5 ± 0.1	1.0 ± 0.3	1.7 ± 0.2

	TIF2			RAP250
mERβ2	Box1	Box2	Box3	Box1
K_D (nM)	66.3 ± 23.2	63.7 ± 21.2	120.8 ± 30.5	272.3 ± 82.4
$k_{a1} \times 10^4$ ($M^{-1}\,s^{-1}$)	4.6 ± 1.2	4.5 ± 0.6	3.4 ± 0.3	6.0 ± 0.8
$k_{d1} \times 10^{-3}$ (s^{-1})	6.2 ± 0.8	5.1 ± 0.6	5.6 ± 1.0	32.3 ± 2.6
$k_{a2} \times 10^{-3}$ (s^{-1})	2.0 ± 0.2	1.8 ± 0.1	1.5 ± 0.1	4.5 ± 1.0
$k_{d2} \times 10^{-3}$ (s^{-1})	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	2.3 ± 0.4

[a] The apparent K_D values were obtained from SPR-generated data using the curve fitting analysis program BIAevaluation. The data were best described using a conformational change model according to equation

$$A + B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} AB \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} AB^*$$

The receptor (A) first forms an unstable complex (AB) with the peptide (B) and then undergoes a conformational change that leads to a more stable complex (AB*). $K_D = (k_{d1}/k_{a1})(k_{d2}/k_{a2})$. Values represent the mean ± SD of three independent experiments.

that ERβ2 suppressed the ERα-mediated transcriptional response, one possibility is that ERβ2 acts as a negative regulatory partner of ERα under specific physiological conditions. It has been demonstrated that ERβ1 acts as modulator of ERα-mediated gene transcription in mouse uterus (*29*). Furthermore, it was found that the ERβ2 protein is upregulated and the colocalization of ERβ2 with ERα is increased during the lactation period in rat mammary gland, indicating a possible role of ERβ2 as a dominant repressor of ERα (*13*). Our study and those of others demonstrate that the two receptor isoforms show differences in response to estradiol and other ligands. It is thus also possible that regulation of receptor isoform expression could govern the tissue effects of specific estrogenic agents. The other possibility is that ERβ2 provides a cellular mechanism to respond to elevated estradiol levels, e.g., achieved in the ovary during pregnancy or the periovulatory phase, higher than those required to fully activate ERα and ERβ1. In addition, it cannot be excluded that there exist phyto- or xenoestrogens that act preferentially at ERβ2. ERβ2 may also have other functions. For example, it was shown that, in the presence of estradiol or raloxifene, ERβ2 can activate the transforming growth factor β promoter as efficiently as ERα and ERβ1 (*11*).

In mouse and rat, the tissue distribution and/or the relative levels of ERβ1 and ERβ2 mRNA seem to be quite different, that is, high expression in liver, pancreas, uterus, breast, and brain for ERβ2 and in nervous system for ERβ1 (*30*),

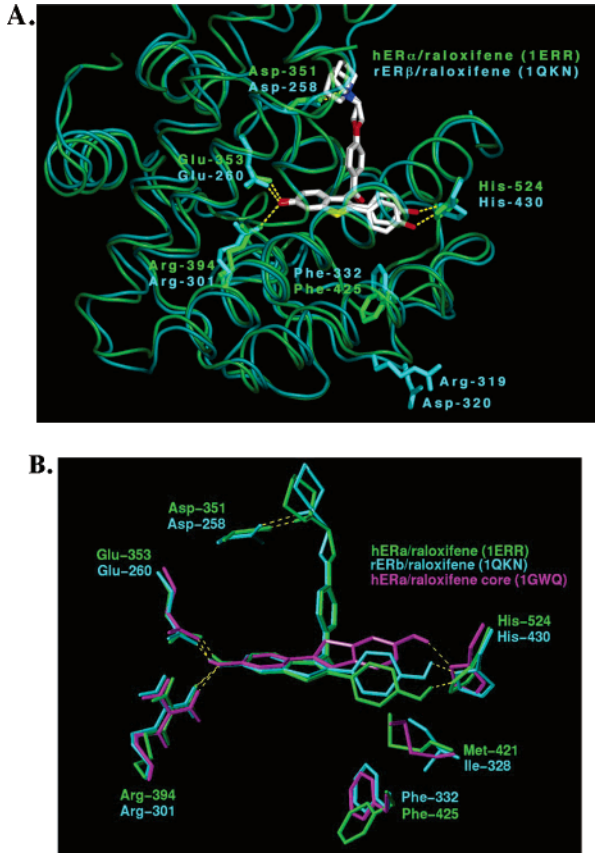


FIGURE 6: (A) An overlay of hER/raloxifene (green, PDB accession code 1ERR) and rERβ1/raloxifene (cyan, 1QKN) crystallographic structures. Critical amino acid side chains (green = ERα and cyan = ERβ) and raloxifene ligand atoms (white = carbon, red = oxygen, blue = nitrogen, and yellow = sulfur) are depicted as tubes. Hydrogen-bonding interactions between the ligands and receptors are shown as dashed yellow lines. The conformation of Phe-425 (ERα)/Phe-322 (ERβ1) affects the positioning of the 2-aryl group of raloxifene and consequently the strength of the hydrogen bond between His-524 (ERα)/His-430 (ERβ1) of the receptor and the 4′-hydroxyl group of the ligand. The close proximity of the 18 aa insert between Arg-319 and Asp-320 in ERβ2 to Phe-322 may influence the preferred conformation of Phe-322, providing a possible explanation for the selectivity of raloxifene for ERβ2 vs ERβ1 (see text for further discussion). This figure was produced using the PyMOL program (*34*). **(**B) An overlay of hERα/raloxifene (green, PDB accession code 1ERR), rERβ1/raloxifene (cyan, 1QKN), and the hERα/raloxifene core (magenta, 1GWQ) crystallographic structures.

implying a specific mechanism regulating expression of one or the other splice variant. In humans, tissue-specific expression of the ERα gene has been shown to be regulated by multiple promoters (*31*). Further studies, including cloning and characterization of putative promoters, should help to elucidate the regulatory mechanisms of ERβ isoform expression. Interestingly, the expression of ERβ2 mRNA has been demonstrated in various human cancer cell lines (*9*). A variant of ERα, analogous to ERβ2, which contains an in-frame insertion between exons 5 and 6 that encodes an additional 23 amino acids in the LBD has been reported in human breast cancers (*32*). In breast, raloxifene acts as a classical antiestrogen to inhibit the growth of mammary carcinoma (*33*). Our findings that raloxifene binds preferentially to ERβ2 imply that the varying expression of ERβ isoforms under certain conditions might have important pharmacological implications.

Selective estrogen receptor modulators (SERMs) are developed to display either agonist or antagonist activity in a tissue-selective manner and are likely to provide novel and improved therapeutic strategies. Although the mechanisms by which these ligands accomplish tissue-selective activity remain to be fully elucidated, multiple ERs provide opportunities for the synthesis of receptor-selective ligands as one class of SERMs. However, isoform-dependent ligand selectivity, reported for the two rodent ERβs in this study, needs to be considered when novel compounds are tested in animal studies.

REFERENCES

1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., et al. (1995) The nuclear receptor superfamily: the second decade, *Cell 83*, 835−839.
2. Tsai, M. J., and O'Malley, B. W. (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members, *Annu. Rev. Biochem. 63*, 451−486.
3. Fawell, S. E., Lees, J. A., White, R., and Parker, M. G. (1990) Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor, *Cell 60*, 953−962.
4. Beato, M., and Sanchez-Pacheco, A. (1996) Interaction of steroid hormone receptors with the transcription initiation complex, *Endocr. Rev. 17*, 587−609.
5. Torchia, J., Glass, C., and Rosenfeld, M. G. (1998) Co-activators and co-repressors in the integration of transcriptional responses, *Curr. Opin. Cell Biol. 10*, 373−383.
6. Tremblay, G. B., Tremblay, A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Labrie, F., and Giguere, V. (1997) Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta, *Mol. Endocrinol. 11*, 353−365.
7. Lu, B., Leygue, E., Dotzlaw, H., Murphy, L. J., Murphy, L. C., and Watson, P. H. (1998) Estrogen receptor-beta mRNA variants in human and murine tissues, *Mol. Cell. Endocrinol. 138*, 199−203.
8. Chu, S., and Fuller, P. J. (1997) Identification of a splice variant of the rat estrogen receptor beta gene, *Mol. Cell. Endocrinol. 132*, 195−199.
9. Hanstein, B., Liu, H., Yancisin, M. C., and Brown, M. (1999) Functional analysis of a novel estrogen receptor-beta isoform, *Mol. Endocrinol. 13*, 129−137.
10. Petersen, D. N., Tkalcevic, G. T., Koza-Taylor, P. H., Turi, T. G., and Brown, T. A. (1998) Identification of estrogen receptor beta2, a functional variant of estrogen receptor beta expressed in normal rat tissues, *Endocrinology 139*, 1082−1092.
11. Lu, B., Leygue, E., Dotzlaw, H., Murphy, L. J., and Murphy, L. C. (2000) Functional characteristics of a novel murine estrogen receptor-beta isoform, estrogen receptor-beta 2, *J. Mol. Endocrinol. 25*, 229−242.
12. Maruyama, K., Endoh, H., Sasaki-Iwaoka, H., Kanou, H., Shimaya, E., Hashimoto, S., Kato, S., and Kawashima, H. (1998) A novel isoform of rat estrogen receptor beta with 18 amino acid insertion in the ligand binding domain as a putative dominant negative regular of estrogen action, *Biochem. Biophys. Res. Commun. 246*, 142−147.
13. Saji, S., Sakaguchi, H., Andersson, S., Warner, M., and Gustafsson, J.-Å. (2001) Quantitative analysis of estrogen receptor proteins in rat mammary gland, *Endocrinology 142*, 3177−3186.
14. Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily, *Science 270*, 1354−1357.
15. Caira, F., Antonson, P., Pelto-Huikko, M., Treuter, E., and Gustafsson, J.-Å. (2000) Cloning and characterization of RAP250, a novel nuclear receptor coactivator, *J. Biol. Chem. 275*, 5308−5317.
16. Jones, C. D., Jevnikar, M. G., Pike, A. J., Peters, M. K., Black, L. J., Thompson, A. R., Falcone, J. F., and Clemens, J. A. (1984) Antiestrogens. 2. Structure−activity studies in a series of 3-aroyl-2-arylbenzo[*b*]thiophene derivatives leading to [6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thien-3-yl]-[4-[2-(1-piperidinyl)ethoxy]-phenyl]methanone hydrochloride (LY 156758), a remarkably

effective estrogen antagonist with only minimal intrinsic estrogenicity, *J. Med. Chem. 27*, 1057−1066.
17. Leygue, E., Dotzlaw, H., Lu, B., Glor, C., Watson, P. H., and Murphy, L. C. (1998) Estrogen receptor beta: mine is longer than yours?, *J. Clin. Endocrinol. Metab. 83*, 3754−3755.
18. Saji, S., Jensen, E. V., Nilsson, S., Rylander, T., Warner, M., and Gustafsson, J.-Å. (2000) Estrogen receptors alpha and beta in the rodent mammary gland, *Proc. Natl. Acad. Sci. U.S.A. 97*, 337−342.
19. Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., and Gustafsson, J.-Å. (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta, *Endocrinology 139*, 4252−4263.
20. Cheng, Y., and Prusoff, W. H. (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction, *Biochem. Pharmacol. 22*, 3099−3108.
21. Rich, R. L., Hoth, L. R., Geoghegan, K. F., Brown, T. A., LeMotte, P. K., Simons, S. P., Hensley, P., and Myszka, D. G. (2002) Kinetic analysis of estrogen receptor/ligand interactions, *Proc. Natl. Acad. Sci. U.S.A. 99*, 8562−8567.
22. Leers, J., Treuter, E., and Gustafsson, J.-Å. (1998) Mechanistic principles in NR box-dependent interaction between nuclear hormone receptors and the coactivator TIF2, *Mol. Cell. Biol. 18*, 6001−6013.
23. Warnmark, A., Almlof, T., Leers, J., Gustafsson, J.-Å., and Treuter, E. (2001) Differential recruitment of the mammalian mediator subunit TRAP220 by estrogen receptors ERalpha and ERbeta, *J. Biol. Chem. 276*, 23397−23404.
24. Wurtz, J. M., Bourguet, W., Renaud, J. P., Vivat, V., Chambon, P., Moras, D., and Gronemeyer, H. (1996) A canonical structure for the ligand-binding domain of nuclear receptors, *Nat. Struct. Biol. 3*, 87−94.
25. Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J.-Å., and Nilsson, S. (1998) Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists, *Mol. Pharmacol. 54*, 105−112.
26. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J.-Å., and Carlquist, M. (1997) Molecular basis of agonism and antagonism in the oestrogen receptor, *Nature 389*, 753−758.
27. Pike, A. C., Brzozowski, A. M., Hubbard, R. E., Bonn, T., Thorsell, A. G., Engstrom, O., Ljunggren, J., Gustafsson, J.-Å., and Carlquist, M. (1999) Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist, *EMBO J. 18*, 4608−4618.
28. Warnmark, A., Treuter, E., Gustafsson, J.-Å., Hubbard, R. E., Brzozowski, A. M., and Pike, A. C. (2002) Interaction of transcriptional intermediary factor 2 nuclear receptor box peptides with the coactivator binding site of estrogen receptor alpha, *J. Biol. Chem. 277*, 21862−21868.
29. Weihua, Z., Saji, S., Makinen, S., Cheng, G., Jensen, E. V., Warner, M., and Gustafsson, J.-Å. (2000) Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus, *Proc. Natl. Acad. Sci. U.S.A. 97*, 5936−5941.
30. Lewandowski, S., Kalita, K., and Kaczmarek, L. (2002) Estrogen receptor beta. Potential functional significance of a variety of mRNA isoforms, *FEBS Lett. 524*, 1−5.
31. Flouriot, G., Griffin, C., Kenealy, M., Sonntag-Buck, V., and Gannon, F. (1998) Differentially expressed messenger RNA isoforms of the human estrogen receptor-alpha gene are generated by alternative splicing and promoter usage, *Mol. Endocrinol. 12*, 1939−1954.
32. Murphy, L. C., Wang, M., Coutt, A., and Dotzlaw, H. (1996) Novel mutations in the estrogen receptor messenger RNA in human breast cancers, *J. Clin. Endocrinol. Metab. 81*, 1420−1427.
33. Purdie, D. W., and Beardsworth, S. A. (1999) The selective oestrogen receptor modulation: evolution and clinical applications, *Br. J. Clin. Pharmacol. 48*, 785−792.
34. DeLano, W. L. (2002) The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA (http://www.pymol.org).

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