A new level of complexity has recently been added to estrogen signaling with the identification of a second estrogen receptor, ERβ. By screening a rat prostate cDNA library, we detected ERβ as well as a novel isoform that we termed ERβ2. ERβ2 contains an in-frame inserted exon of 54 nucleotides that results in the predicted insertion of 18 amino acids within the ERβ hormone-binding domain. We also have evidence for the expression of both ERβ1 and ERβ2 in human cell lines. Competition ligand binding analysis of bacterially expressed fusion proteins revealed an 8-fold lower affinity of ERβ2 for 17β-estradiol (E2) [dissociation constant (Kd); 8 nM] as compared with ERβ1 (Kd; 1 nM). In vitro transcribed and translated ERβ1 and ERβ2 bind specifically to a consensus estrogen responsive element in a gel mobility shift assay. Furthermore, we show heterodimerization of ERβ1 and ERβ2 with each other as well as with ERα. In affinity interaction assays for proteins that associate specifically with the hormone-binding domain of these receptors, we demonstrate that the steroid receptor coactivator SRC-1 interacts in an estrogen-dependent manner with ERα and ERβ1, but not with ERβ2. In cotransfection experiments with expression plasmids for ERα, ERβ1, and ERβ2 and an estrogen-responsive element-containing luciferase reporter, the dose response of ERβ1 to E2 was similar to that of ERα although the maximal stimulation was approximately 50%. In contrast, ERβ2 required 100- to 1000-fold greater E2 concentrations for maximal activation. Thus, ERβ2 adds yet another facet to the possible cellular responses to estrogen. (Molecular Endocrinology 13: 129–137, 1999)

INTRODUCTION

The estrogen receptor (ER) is a member of the nuclear receptor superfamily of ligand-activated transcription factors that include receptors for steroid hormones, thyroid hormone, vitamin D, retinoic acid, and eicosanoids (1–6). After diffusion into the cell, estradiol binds to the ER, leading to ER dimerization followed by binding to a conserved estrogen responsive element (ERE) in the regulatory region of target genes. Amino acid sequence comparison of the ER with other nuclear receptors has shown that the receptor is composed of conserved functional regions. The N-terminal transactivating region (AF1) is able to activate transcription in a hormone-independent manner, and this region has been shown to be a target of the mitogen-activating protein kinase-regulatory pathway (7). The DNA-binding domain enables the receptor to bind to its specific DNA target, the ERE. The consensus ERE consists of a palindrome of the half-site sequence 5'-GGTCA-3' separated by 3 bp. The AF2 domain is overlapped by the hormone-binding domain (HBD) and activates transcription in response to estrogen or synthetic estrogen agonists (8).

The mechanism of transactivation by nuclear receptors has recently achieved further complexity by the discovery of an increasing number of coregulators. This group of coregulators can be subdivided into coactivators, corepressors, and integrators. The coactivators were initially biochemically identified as ERAP160 and 140 and RIP160, 140, and 80 (9, 10) by their ability to specifically interact with the HBD of the receptor in a ligand-dependent manner. Specifically for the ER, this interaction was promoted by E2 but antiestrogens such as 4-OH-tamoxifen were able to effectively block this interaction. Yeast two-hybrid screening led to the molecular cloning of the steroid receptor coactivator (SRC) 1, which when cotransfected with nuclear receptors, including ER, was capable of augmenting ligand-dependent transactivation (11). Subsequent cloning and sequence comparison of transcriptional intermediary factor (TIF)2 and glucocorticoid receptor interacting protein (GRIP)1 revealed GRIP1 to be the mouse homolog of human TIF2. More recently, p300/CBP cointegrator protein (p/CIP) [also receptor-associated coactivator (RAC)3 (12) and activator for thyroid hormone and retinoid receptors (ACTR) (13)] were shown to be new members of this family (14). Interestingly, this ER coactivator was also identified as amplified in breast cancer (A1B) (15).
addition, the phospho-CREB-binding protein CBP and the related p300 have been demonstrated to be ER-associated proteins and involved in ligand-dependent transactivation (16, 17). In contrast to the coactivators mentioned above, these proteins are targets of signals mediated by a variety of distinct pathways. Moreover, by interaction with components of the basal transcription machinery, these proteins are thought of as integrators of signals from these diverse pathways. This increasing number of coregulatory factors has added immensely to our understanding of how steroids such as E₂ are able to alter the expression of specific genes at the molecular level.

Recently, a new member of the nuclear receptor family with high homology to ER was cloned from rat, mouse, and human and was termed ERβ (18–20). The homology to the rat ER protein (now ERα) was shown to be 95% in the DNA-binding domain and 55% in the HBD (18). In situ hybridization studies in rat revealed a prominent expression of this novel receptor in the epithelial cells of the secretory alveoli of the prostate and the granulosa cells of the primary, secondary, and mature follicles of the ovary. ERβ was found to bind E₂ with high affinity and in transient transfection experiments ERβ was capable of activating transcription of a reporter gene in an estrogen-dependent manner.

Recently, a partial clone for an alternative splice variant of ERβ2 has been described (21). Here we report the complete cloning and functional analysis of this novel rat ERβ isoform.

RESULTS

Cloning of ERβ2, an Alternative Splice Variant of ERβ

To obtain clones of ERβ we screened a λgt11 rat prostate cDNA library with two oligonucleotide probes derived from the published ERβ sequence, corresponding to the nucleotides 418–477 and 1248–1307. Primary screening of ~900,000 phage revealed four positive plaques that were confirmed in a secondary screening and isolated in a tertiary screening. Inserts of all independently isolated plaques were then subcloned and subjected to nucleotide sequencing. The full-length cDNA clone diverged from the previously published rat cDNA at two positions (496 T to A; 729 C to G). These nucleotide changes result in amino acid changes that are conserved in the published sequence of human ERβ. Therefore, it is likely that these result from polymorphisms in the ER. More interestingly, three of four independent clones revealed an insertion of 54 nucleotides at position 1374 of the previously published rat cDNA (Fig. 1). This results in an in-frame insertion of 18 amino acids in the predicted HBD of this receptor. We therefore termed this alternative splice variant ERβ2, ERβ1 being the originally published sequence. The amino acid sequence of this insert exhibits no homology to known proteins or peptide motifs when computer database searches were performed. Recently, the sequence of ERβ2 was also reported as an ERβ splice variant (21).

Expression of ERβ1 and ERβ2 in Different Human Cancer Cell Lines

Since screening of the prostate cDNA library revealed that the majority of clones (3/4) encode ERβ2, we examined the expression pattern of this nuclear receptor splice variant in a variety of human cancer cell lines derived from breast, uterus, ovary, and prostate tissue. We isolated RNA from these cells, subjected it to RT using oligo dT primers, and performed PCR reactions with primers derived from the human ERβ sequence flanking the insertion site of the 18 amino acids (aa) in the ERβ sequence. ERα-specific primers and primers from the β2-microglobulin gene were used to control for the quality of the cDNA. As shown in Fig. 2, as expected, all cDNAs revealed a product for the ubiquitously expressed β2-microglobulin (lower panel). The ERα transcript was detectable in breast cancer cell lines previously described as ERα-positive such as MCF7, T47D, and BT-20 as well as in the endometrial cancer cell line ECC1 and in the ovarian cancer cell line OVCAR-3. When the PCR reaction was performed with ERβ-specific primers, a band corresponding to the expected size for the previously published ERβ sequence was detectable in the breast cancer cell lines BT-20, MDA-MB231, and T47D and in primary normal human mammary epithelial cells (HMECs). Moreover, a transcript for ERβ1 was detectable in the ovarian cancer cell lines OVCAR-3 and UPN36T. Among endometrium cancer cell lines tested, only the ERα-negative Ishikawa cell line showed an ERβ1 transcript. The human prostate cancer cell lines, PC-3 and DU145, were also positive for ERβ1 expression. To confirm that the bands indeed correspond to ERβ, we transferred the PCR products onto nitrocellulose and subjected it to Southern blot analysis with a radiolabeled nucleotide probe derived from the previously pub-
lished ERβ sequence. As shown in Fig. 2, the band of ~340 bp was indeed reactive with this probe. Interestingly, using PCR primers flanking the insertion in the rat cDNA also revealed a band with the expected size for ERβ2 in the human ovarian cancer cell line OVCAR-3 and the osteosarcoma cell line U2OS. To confirm the origin of this PCR product as ERβ2, we probed duplicate blots with an oligonucleotide probe corresponding to the unique 54 nucleotides of the ERβ2 sequence as shown in Fig. 2, top panel. These data indicate that although ERβ1 is present in a variety of different cells, the ERβ2 transcript is restricted to a minority of these cell lines, suggesting a specific mechanism regulating expression of these splice variants.

ERβ2 Binds Estradiol with Lower Affinity Than ERβ1

Since insertion of the 18 aa in ERβ2 occurs in the predicted hormone-binding domain of this receptor, we first examined the binding affinity of estradiol for ERβ1 and ERβ2. Therefore, [3H]E2 was used to conduct competition ligand-binding studies of ERβ1 and ERβ2. Using bacterial expressed glutathione S-transferase (GST) fusion proteins containing the HBDs of both receptors, dissociation constants (Kd) for E2 were 1 nM for ERβ1 but 8-fold higher (Kd = 8 nM) for ERβ2 (Fig. 3). These data indicate that the 18-aa insertion in the HBD of ERβ2 lowers its affinity for E2.

ERβ2 Binds to an ERE

It has been previously shown that ERβ1 is capable of binding to a consensus ERE with high affinity. To test whether the 54-nucleotide insertion of ERβ2 had an influence on DNA binding, we conducted electrophoretic mobility shift assays (EMSAs). Receptors were expressed in vitro to comparable levels (data not shown). As demonstrated in Fig. 4, both ERβ1 and

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**Fig. 2.** Expression of ERα, ERβ1, and ERβ2 in Various Human Cancer Cell Lines

RNA extracted from various human cancer cell lines was subjected to RT using oligo dT primers. Cell lines used were the human ovarian cancer cell lines SW626 (lane 1), OVCAR-3 (lane 2), CAOV-3 (lane 3), UPN36T (lane 4), and the human breast cancer cell lines BT-20 (lane 5), MDAMB231 (lane 6), T47D (lane 7), and MCF7 (lane 8), normal HMEC (lane 9), two human endometrium cancer cell lines ECC1 (lane 10) and Ishikawa (lane 11), the human prostate cancer cell lines PC-3 (lane 12), Du145 (lane 13), and LnCAP (lane 14), the green monkey kidney cell line CV-1 (lane 15), and the human osteosarcoma cell line U2OS (lane 16). Integrity of the cDNAs was verified by PCR using primers specific to human β2-microglobulin (β2MG) cDNA (lower panel). Expression of ERα was analyzed using primers specific to the human ERα cDNA (second panel from bottom). To analyze expression of ERβ1 and ERβ2, PCR was performed with primers flanking the alternative splice site. Southern blot analysis on these PCR products was performed using either the ERβ2-specific insert as a probe (top panel) or a probe to the common ERβ region (second panel from the top).

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**Fig. 3.** Estradiol Binds to ERβ1 and ERβ2

E2 binding to GST fusion proteins of either ERβ1 (open squares) or ERβ2 (closed squares) HBD was assayed as described in Materials and Methods. Binding is expressed as the percentage of bound radiolabeled E2 at a given competitor concentration compared with binding of labeled E2 in the absence of competitor.
ERβ2 were able to bind specifically to the ERE probe in a hormone-dependent manner, under the stringent conditions used (22). Specificity of binding was confirmed by the fact that unlabeled ERE could compete for binding of all receptors to the labeled probe (lanes 5), whereas competition with a mutated ERE (lanes 6), or an unrelated AP-1 sequence (lanes 7), did not have an influence on binding.

When EMSA was performed with increasing E2 concentrations (lanes 2–4), it was interesting to note that maximal binding of ERα (to the ERE) occurred at the lowest concentration of E2 used (1 nM) (lane 2), whereas both ERβ1 and ERβ2 showed a dose response with maximal binding at 100 nM. Despite the apparent differences in hormone binding affinity for ERβ1 and ERβ2, both receptors bound with similar affinity to DNA.

**Heterodimerization Occurs between ERα and Both ERβ Isoforms**

Since it has been demonstrated that ERα and ERβ1 are capable of forming heterodimers upon ligand binding, we set forth to investigate whether the newly identified splice variant of ERβ also forms heterodimers with ERα. Therefore, we performed EMSA on a labeled ERE probe using in vitro translated full-length receptors in the presence of 100 nM E2 (Fig. 5).

When ERα (lanes 1 and 2), ERβ1 (lanes 3 and 4), or ERβ2 (lanes 5 and 6) were incubated with the labeled ERE probe, antibodies specific for ERα or ERβ were able to supershift the homodimeric ER/DNA complexes (lanes 2, 4, and 6). When in vitro translated ERα and ERβ1 (lanes 7–10) or ERα and ERβ2 (lanes 11–14) were coincubated with the labeled probe, each antibody was able to shift the protein/DNA complex to a different size than the respective homodimeric receptor (lanes 8, 9, and 12, 13). When both antibodies were coincubated either with ERα/ERβ1 or with ERα/ERβ2, a supershifted band was detected, indicating that indeed ERα forms heterodimers on DNA with ERβ1 and ERβ2 (lanes 10 and 14). Heterodimers could be detected at E2 concentrations as low as 1 nM (data not shown).

**Interaction of SRC1 with ERα and ERβ1, but Not ERβ2**

The role of putative coactivators in the hormone-dependent transcriptional regulation by ERβ1 and ERβ2 was addressed by affinity purification of ERβ1- and ERβ2-associated proteins. The HBD of these receptors was fused to GST and used to purify proteins from the metabolically labeled human breast cancer cell line MCF-7 (Fig. 6), the human breast cancer cell line MDA-MB231, and the human osteosarcoma cell line U2OS (data not shown). In the case of ERα (lanes 1–3), previously described proteins bound GST-HBD-ERα in the presence of E2 (lane 2), but not in its absence (lane 1) nor in the presence of the ER antagonist tamoxifen (lane 3). Using the GST-HBD-ERβ1 fusion protein as an affinity matrix (lanes 4–6), only the...
ERAP160/SRC1 family of proteins could be detected (lane 5). Interestingly, when performing this experiment with the same amount of GST-HBD-ERβ2, we were unable to detect associated proteins that specifically interact with ERβ2 (lane 8). As ERβ2 ligand-binding analysis had detected an 8-fold lower affinity for E_2, we conducted the same experiment using increasing amounts of estradiol (1 μM and 10 μM). Again, using GST-HBD-ERβ2 as an affinity matrix, we were unable to detect proteins interacting with the receptor in an estradiol-dependent manner (data not shown). To exclude the possibility that other domains of ERβ2 were required for coactivator interaction, we performed GST pull-down experiments using full-length ERβ1 and ERβ2 as GST fusion proteins and obtained similar results (data not shown). To test whether the proteins in the 160-kDa range, detected in this assay to bind to ERα and ERβ1, include the cloned steroid receptor coactivator SRC1, we performed Western blot analysis with an anti-SRC1-specific antibody on proteins associating with the ligand-binding domain of these receptors (Fig. 7A). For this purpose we used whole-cell extracts from MCF-7 cells. Cell lysates were incubated with the GST-HBD affinity matrices of the different receptors in the absence or presence of increasing amounts of E_2 (1 μM and 10 μM) or 4-OH-Tamoxifen (1 μM). Specifically bound proteins were resolved by SDS-PAGE and transferred onto nitrocellulose. Immunoblotting with a monoclonal antibody directed against human SRC1 revealed significant SRC1 binding only for GST-HBD-ERβ1 and GST-HBD-ERβ2 in the presence of E_2 but not for GST-HBD-ERβ2 (Fig. 7A). Negative control samples consisting of glutathione-sepharose or GST immobilized on glutathione sepharose did not show binding (data not shown). To confirm these results, we tested the ability of recombinant SRC1 to interact with ERβ1 or ERβ2. Again, in vitro synthesized [35S]methionine-labeled SRC1 associated specifically in an E_2-dependent manner only with GST-HBD-ERα (lane 2) and GST-HBD-ERβ1 (lane 5) but not GST-HBD-ERβ2 (lane 8) (Fig. 7B).

Transcriptional Activity of ERβ2 on an ERE

To investigate whether an ERβ can mediate transcriptional activity of ERβ2, we used a luciferase reporter gene containing two copies of an ERE and the viral thymidine kinase promoter, driving the expression of the luciferase reporter gene for transactivation studies. We performed cotransfection experiments in which U2OS cells were transfected with either an expression plasmid for ERα, ERβ1, or ERβ2 and the estrogen-responsive reporter gene construct. E_2 maximally stimulates ERα-mediated transactivation through an ERE of approximately 6-fold at a doses as low as 0.1 nM (Fig. 8). ERβ1 was less potent in stimulating transcription through an ERE with only 50% of the maximal level seen with ERα, although transcriptional activation occurs with a similar dose response. In contrast, when the same experiment was performed with an expression plasmid for ERβ2, E_2 failed to stimulate transactivation through the ERE at 0.1 nM or 1 nM E_2 (Fig. 8). Increasing doses of E_2 to 100 nM were able to stimulate ERβ2-mediated activation to a level comparable ERβ1 (Fig. 8). These data indicate that ERβ2 requires 100- to 1000-fold higher concentrations of E_2 to stimulate transcription to the same extent as ERβ1.

DISCUSSION

Estradiol mediates its diverse biological effects by binding to the ER, thereby allowing the receptor to
bind to and activate transcription through estrogen-responsive elements in the promoter region of target genes. The phenotype of ERα knockout mice pointed to the potential existence of an alternative mediator for E2 action (23). Recently, a new member of the nuclear receptor family with very high homology to ERα was cloned and termed ERβ (18). The identification of ERβ has added a new level of complexity to E2 signaling.

In this report we describe the cloning of an alternative splice variant of ERβ, ERβ2, which contains an 18-aa insert in the predicted hormone binding domain of this nuclear receptor, adding yet another level of complexity to E2 signaling. We have demonstrated that this isoform is expressed in normal rat prostate as well as various human cancer cell lines. The fact that in some cell lines one isoform appears to be more prominent than the other, and that this relative ratio varies from tissue to tissue examined, suggests a specific mechanism regulating expression of one or the other splice variant.

Interestingly, we demonstrate that both receptors coexist in certain cells and can heterodimerize on the ERE. Further complexity is achieved, since not only do ERβ1 and ERβ2 heterodimerize, but each can also heterodimerize with ERα. Since we could not demonstrate that ERβ2 binds the coactivator SRC1 one could speculate that heterodimerization of ERα or ERβ1 with ERβ2 regulates the recruitment of this coactivator to the transcriptional complex. Since previous studies have demonstrated that other transcriptional cointegrators, namely p300 and the phospho-CREB binding protein (CBP) appear to be rate limiting for active transcription (16), this reduction of SRC1 recruitment might reduce transcriptional activity through the ERE.

Another recent study has shown that while both ERα and ERβ1 activate E2-mediated transcription through an ERE, they exert opposite effects through an AP-1 site (24). While E2 stimulates ERα-mediated transcription through an AP-1 site, E2 inhibits ERβ1-mediated transcription through the same response element. It still has to be demonstrated which effect ERβ2 mediates through an AP-1 site, and which effect the different heterodimers of these receptors mediate through this response element in the presence of different ligands.

Interestingly, alignment of the ERα sequence with ERβ, as compared with the predicted structure of nuclear receptors, indicates that the 18-aa insert in ERβ2 lies in helix 6 of this receptor. This relatively nonconserved region among different nuclear receptors follows immediately after the α-turn within the ligand binding domain of the receptor (25). The addition of 18 aa in this region might distort the correct conformation of this receptor for high-affinity E2 binding as supported by our E2 binding data. High physiological E2 concentrations achieved especially in the ovary during pregnancy or the peri-ovulatory phase might be sufficient to activate ERβ2. Another interesting possibility is that this insertion creates a new conformational change required for high-affinity binding of a yet unidentified ligand other than E2.

With respect to the basic mechanisms by which nuclear receptors initiate transcription of their target genes, much of recent research in the field has focused on so-called coactivators of these proteins, which bind the nuclear receptors in a ligand-dependent manner to augment AF-2-mediated transactivation. The coactivators have been identified by the in vitro interaction of the ligand-binding domain of ERα fused to GST as an affinity matrix for proteins interacting in an E2 dependent manner (9, 10). Using the same approach we could demonstrate the interaction of the coactivator of nuclear receptors, SRC1 with both ERα and ERβ1. Interestingly, GST fusion proteins of both the ligand-binding domain and the full-length ERβ2 failed to interact with SRC1 in a ligand-dependent manner, despite the fact that both fusion proteins were able to bind both E2 and an ERE. It is striking that this results in a shift in the dose response of ERβ2 to E2 but not the maximal level of activation. This suggests the possibility that under certain conditions ERβ2 might act to dampen cellular responses to estrogen.

We and others (29) have identified an alternative splice variant of ERβ termed ERβ2. This protein exhibits interesting properties as a mediator of estrogen action and provides new complexity to the spectrum of potential cellular responses to estrogen.

MATERIALS AND METHODS

Library Screening and Plasmids

We screened a λgt11 rat prostate cDNA library (CLONTECH, Palo Alto, CA), according to the manufacturers guidelines.
with two radiolabeled oligonucleotides corresponding to nucleotides 418–477 and 1248–1307 of the previously published rat ERα sequence. Labeling of the probe was performed with γ-32P-ATP (6000 mCi/mmol; New England Nuclear, Boston, MA) in the presence of T4 polynucleotide kinase according to standard procedures (26). Positive plaques from the primary screening were isolated by secondary and tertiary screening, and phage DNA was obtained by boiling plaques in 100 ml H2O. Plaque DNA was then amplified by PCR using ERα-specific primers corresponding to nucleotides 402–419 and 1866–1885 of the ERβ sequence. One plaque contained the full-length ERβ cDNA. The resulting PCR fragment was blunt ended using T4 DNA polymerase and was subcloned into the EcoRV site of pcDNA3.0 (Invitrogen, San Diego, CA), resulting in pERβ1 and was completely sequenced from both strands by automated sequence analysis (ABI 3000, Molecular Biology Core Facility, Dana Farber Cancer Institute). DNA from the remaining plaques yielded PCR products when a 5′-primer corresponding to nucleotides 922–941 of the ERα was used for amplification, indicating that these were partial cDNA clones. Amplification in pGEX ERα (Promega, Madison, WI) resulted in pERβ2, resulting in pGEX ERβ2HBD. These plasmids were also subjected to complete nucleotide sequence analysis, revealing the alternative splice variant in three of these clones. To obtain the full-length ERβ2 cDNA we liberated the ERβ1 cDNA from pERβ1 by EcoRI/NcoI digest and subcloned the 1.4-kb insert into the corresponding sites of pBluescript SK− (Invitrogen), resulting in pBS ERβ1. This subclone was confirmed by partial sequence analysis. The 3′-sequence of ERβ2 was then liberated from the pGEX2TK ERβ2 plasmid by EcoRI digest, blunt ending, and Smal digest and subcloned into NotI-digested, blunt ended, and then Smal-digested pBSERβ1, resulting in pBSERβ2. Correct orientation of the 3′-end was confirmed by restriction analysis and confirmed by partial sequence analysis, also revealing the absence of the 5′-nucleotide insert. To generate eukaryotic expression plasmids for ERβ1 and ERβ2, the corresponding cDNAs were liberated from pBSERβ1 and ERβ2 by SacII digest, blunt ending followed by XhoI digest, and subcloned into NotI-digested, blunt ended, and then XhoI-digested pcDNA 3.1(−) vector (Invitrogen), resulting in pcDNA ERβ1 and pcDNA ERβ2. To obtain full-length GST fusion proteins of ERβ1 and ERβ2 the corresponding cDNAs were PCR amplified with primers

5′-GGTGGATCCCGAGCAGTACGTCGGCCGCTG-3′ 5′-GGATGAATTCCTGAGAAGCTGTAGGTTCC-3′ using pBSERβ1 and ERβ2 as templates, BglII/EcoRI digested, and subcloned into BamHI/EcoRI-digested pGEX2TK plasmid, resulting in pGEX ERβ1fl and pGEX ERβ2fl. Both plasmids were verified by complete nucleotide sequence analysis.

**Cells and Cell Culture**

Cell lines MCF-7, BT-20, MDAMB231, T47D, ECC1, Ishikawa, PG-3, Du145, LnCAP, CV1, and U2OS were obtained from American Type Culture Collection (Manassas, VA). The normal HMECs were purchased from Clonetics (San Diego, CA). The human ovarian cancer cell lines Sw626, OVCAR-3, CAOV-3, and UPN36T were a gift from Dr. S. Cannistra. Cells were maintained in DMEM containing 10% FBS (vol/vol) (Sigma Chemical Co., St. Louis, MO) at 37 C and 5% CO2/95% air.

**RT-PCR and Southern Blot**

RNA extraction was performed using the Ultraspec RNA isolation system (Biotecx, Houston, TX) according to manufacturer’s guidelines. RT was performed using oligo dT primers. Using the Gibco BRL RT Kit (GIBCO BRL, Grand Island, NY). Two micrograms of cDNA were used as a template for PCR reactions using two microglobulin-specific primers (CLONTECH, primers specific to human ER (5′-GGGAGCTGGTTCACTAGTCGATC-3′ and 5′-GTCGACACTGTGAGGTGGATGATG-ATG-3′) or primers specific to human ER (5′-GCCTCCATGATGATGCTGCTG-3′ and 5′-GATGACCTGTGGATCAGCAAGAGG-3′). PCR cycling was performed using a touch down program: 1) 95 C, 1 min; 2) 95 C, 30 sec; 60 C, 30 sec (−0.5 C/cycle); 72 C, 2 min; 3) 72 C, 2 min, 4 C, for ever, 35 cycles, 60 C to 40 C. Resulting PCR products were subjected to electrophoresis in 2% agarose gels. In case of ER, products were transferred to nitrocellulose and probed with end-labeled oligonucleotides either common to ERβ1 and ERβ2.

**Ligand Competition Analysis**

For ligand competition studies GST HBd fusion proteins of ERβ1 and ERβ2 were diluted in HED buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol) and incubated with 1 nM [3H]-E2 and various concentrations of unlabeled diethylstilbestrol. The bound and unbound estrogens were separated using dextran-coated charcoal (28). The amount of bound [3H]-E2 was presented as a percent of total bound in the absence of diethylstilbestrol.

**Gel Mobility Shift Assay**

Recombinant ERα, ERβ1, and ERβ2 cDNAs were transcribed and translated in vitro in TNT-T7 coupled rabbit reticulocyte lysates (Promega, Madison, WI) from the T7 Promoter following the manufacturer’s guidelines. Typically 4 ml of programmed lysate (or 4 ml of a 1:50 dilution of the ERβ1 GST-fusion protein for ERβ1 and ERβ2 heterodimers) were used in each binding reaction. The binding reactions were carried out in binding buffer A100 (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 1 mM dithiothreitol), 0.5 mg of poly deoxynosinosic-deoxyctydillic acid, 20 mg BSA, 4 mM H2O2, 7.5 mM MgCl2 (final concentration), and 4 ng of probe that was labeled by end-filling with Klenow in the presence of [32P]α-dGTP. Preincubations containing ligand, antibody, and/or cold competitor as indicated were performed at room temperature for 20 min. After the incubation step the probe was added and binding was conducted for 15 min at room temperature. The entire reaction of 17 ml was loaded onto a 4% gel, and electrophoresis was carried out at 110 V for 2 h at room temperature. Gels were dried and exposed for 2–5 h at −80 C. The following antibodies were used: AER 314 (Neomarkers, Fremont, CA), mouse polyclonal serum for ERβ1 and ERβ2. We used the following oligonucleotides and their compliments as probes and competitors:

ERE, 5′-GATCTTCTAGTCGACTGACTGGCCGACCTGGAGCCTGG-3′;

mERE, 5′-GATCTTCTTTAGTCGACGAGCGACAGTCCGAGCCGACTGGCCTGGG-3′;

AP1, 5′-GAATGTGACTGTGTCTGCAGACGAGCGACAGTCCGAGCCGACTGGCCTGGG-3′.
Metabolic Labeling and Protein-Protein Interaction Assay

Before the metabolic labeling, MCF-7 cells were preincubated with methionine-free DMEM for 10–20 min. Confluent 150-mm diameter dishes were labeled with 1 mCi (1 Ci = 37 GBq) [35S]methionine (New England Nuclear, Boston, MA) for 4 h in methionine-free DMEM. After labeling cells were washed extensively with ice-cold PBS and lysed in 1 ml of buffer A (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 0.5% Nonidet P-40). After 30 min of rotation at 4 °C, cell extracts were clarified by centrifugation at 12,000 rpm, and the supernatant was collected in a fresh tube. Lysates containing 2.5 × 10^7 cpm were then incubated with a GST fusion protein containing the HBDs of either ERα, ERβ1, or ERβ2 (GST-HBD ERα, ERβ1, or ERβ2) immobilized on 50 ml of glutathione-Sepharose beads in the presence or absence of the appropriate ligand in buffer B (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA) as previously described (9). After washing the beads three times in 1 ml of buffer B and once in 1 ml of buffer A, proteins were eluted in SDS/sample buffer and resolved on 7.5% SDS/PAGE. Gels were fixed in 35% methanol/10% glacial acetic acid, fluorographed in Enhance solution (New England Nuclear), and dried before exposure to film.

Western Blotting

Protein-protein interaction assays were performed as described above using unlabeled cell extracts from MCF-7 cells. Proteins were resolved directly in SDS/polyacrylamide gels after boiling in SDS sample buffer. Immunodetection was performed after blocking the membranes overnight at 4 °C in 20 mM Tris-HCl, pH 7.5, 157 mM NaCl, 0.05% Tween 20, and 5% powdered milk by incubating membranes with an anti-SRC1 antibody for 2 h at room temperature. Monoclonal antibody raised against GST-SRC1 was used for immunoblot analysis in a dilution of 1:100. Specifically bound primary antibody was detected with peroxidase-coupled secondary antibody and chemiluminescence.

In Vitro Transcription and Translation

Recombinant SRC1 cDNA in pBluescript was transcribed and translated in TNT-T3 coupled reticulocyte lysates (Promega, Madison, WI) in the presence of [35S]methionine from the T3 promoter following the manufacturer’s guidelines.

Transient Transfection and Luciferase Assay

For transient transfections U2OS cells were seeded in 24-well plates in phenol red-free DMEM supplemented with 10% charcoal dextran-treated FBS. Cells at a density of 40,000/well were transfected with 100 ng of reporter plasmid, 10 ng of receptor expression vector, 10 ng βActin β-gal plasmid and 680 or 690 ng of salmon sperm DNA to a total of 800 ng using the calcium phosphate/DNA precipitation method. After 16 h, cells were washed once with PBS and were left either untreated or treated with 0.1 mM, 1 mM, 10 mM, or 100 mM E2 for 16 h. For luciferase assays, cells were lysed in potassium phosphate containing 1% Triton X-100. Light emission was detected using a luminometer after addition of luciferin. β-Gal activity was detected using the Galacto-Star (Tropix, Bedford, MA).

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