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Estrogen Receptors α and β Form Heterodimers on DNA*

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The estrogen receptor (ER) is expressed in two forms, ERα and ERβ. Here we show that ERα and ERβ, expressed both *in vitro* and *in vivo*, form heterodimers which bind to DNA with an affinity (K_d of approximately 2 nM) similar to that of ERα and greater than that of ERβ homodimers. Mutation analysis of the hormone binding domain of ERα suggests that the dimerization interface required to form heterodimers with ERβ is very similar but not identical to that required for homodimer formation. The heterodimer, like the homodimers, are capable of binding the steroid receptor coactivator-1 when bound to DNA and stimulating transcription of a reporter gene in transfected cells. Given the relative expression of ERα and ERβ in tissues and the difference in DNA binding activity between ERα/ERβ heterodimers and ERβ it seems likely that the heterodimer is functionally active in a subset of target cells.

Estrogen receptors (ER)[1] were recently shown to be encoded by two distinct genes, *ERα* and *ERβ* (1, 2). Reverse transcription-polymerase chain reaction (PCR) analysis indicates that ERβ is highly expressed in prostate and ovary (1, 2), but moderate expression was detected in many other tissues including testis and uterus, some of which also seem to express ERα (3). The two receptors which share about 95% homology in the DNA binding domain and 55% homology in the ligand binding domain, both bind to a consensus estrogen response element (ERE) (4) and exhibit similar ligand binding properties (3). They are poorly conserved in the N-terminal domain but ERβ, like ERα, appears to contain a similar activation domain, activation function 1 (AF-1) sensitive to a mitogen-activated protein kinase pathway (4–6). In addition, both receptors contain a second activation domain, activation function 2 (AF-2) (7, 8), whose activity is enhanced by the coactivator SRC-1 (4, 9, 10). Thus, although the relative expression of ERα and ERβ varies in cells, their ligand binding, DNA binding, and transactivation properties are rather similar to one another.

Steroid hormone receptors usually bind to inverted DNA repeats as homodimers, although the glucocorticoid and mineralocorticoid receptors have been reported to form heterodimers, at least *in vitro* (11, 12). In the classically accepted model of steroid hormone action, the estrogen receptor is sequestered in an inactive state in a multiprotein complex in the absence of hormone (13). Upon estrogen binding, the receptor forms homodimers which then interact with response elements in the vicinity of target genes and modulate rates of gene transcription. In view of the similarity of the ligand binding domain of ERα and ERβ we investigated the possibility that the two receptors may form functional heterodimers in target cells. ERα and ERβ were capable of forming heterodimers on DNA that could bind the coactivator, SRC-1, and appeared to stimulate transcription of a reporter gene. Moreover, we demonstrate that while the region of ERα required for homodimerization overlaps with that required for heterodimerization the two regions are not coincident.

EXPERIMENTAL PROCEDURES

Plasmids—The isolation and construction of cDNA clones that encode the mouse ERα and a series of point mutants for analyzing receptor dimerization have been described previously (14, 15). To express human ERβ the 1.5-kilobase ERβ cDNA (1) was subcloned into the *Bam*HI site of pSP65 for *in vitro* transcription and translation and pSG5 for mammalian cell expression. The human ERα cDNA from pSG5HEGO, kindly provided by Pierre Chambon, was subcloned into the *Eco*RI site of pSP65. Glutathione *S*-transferase (GST)-SRC-(570–780) was generated by subcloning a PCR fragment of SRC-1 into pGEX2TK.

In Vitro Transcription and Translation—ERα and ERβ protein was synthesized *in vitro* using the TNT-coupled reticulocyte lysate system (Promega) in the presence of 0.1 mM methionine as described previously (16). To quantitate the relative amount of receptors produced, 1 μCi/μl [^{35}S]methionine (Amersham) was included in the reaction mixture.

Transfection Experiments—For biochemical analysis, wild-type and mutant receptors were overexpressed in COS-1 cells by electroporation using a Bio-Rad gene pulser at 450 V and 250 millifarads as described previously (17). Cells were transfected with 20 μg of expression plasmid, either pSG5HEGO or pSG5ERβ as indicated. After 2 days, cells were harvested, and whole cell extracts were prepared using a high salt extraction buffer (400 mM KCl, 20 mM HEPES, pH 7.4, 1 mM dithiothreitol, 20% glycerol, plus protease inhibitors) as described (17). Their protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad). For transient transfection assays, HeLa cells were plated into 24-well microtiter plates in phenol red-free medium containing 5% charcoal/dextran-stripped fetal calf serum. Cells were transfected using a modified calcium phosphate coprecipitation method (18) with 1 μg of pEREBLCAT reporter plasmid, a total of 10 ng of ERα and ERβ expression plasmids as described under "Results," 150 ng of pJ7lacZ plasmid as an internal control, and pSG5 plasmid to a total of 1.5 μg of DNA per well. After 24 h, the cells were washed with Dulbecco's modified Eagle's medium and then maintained in medium with or without 1×10^{-8} M 17β-estradiol for 24 h. The cells were then washed with phosphate-buffered saline and harvested in lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, and 0.65% Nonidet P-40). Extracts were assayed for CAT activity (19) or β-galactosidase activity using a Galacto-Light chemiluminescent assay (Tropix).

DNA Binding Assays—DNA binding was assayed using a gel shift assay. Aliquots of receptors, either translated *in vitro* or expressed in COS-1 cells, were incubated with a ^{32}P-labeled double-stranded oligonucleotide probe containing a consensus ERE sequence (5′-CTAGAAAGTCAGGTCACAGTGACCTGATCAAT-3′) as described previously (17). The human ERα monoclonal antibody H226 or ligands were added as indicated. In some experiments GST-SRC-(570–780), expressed and purified as described previously (20), was added. The

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[1] The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; AF-1, activation function 1; AF-2, activation function 2; GST, glutathione *S*-transferase; MOR, mouse estrogen receptor; PCR, polymerase chain reaction; SRC-1, steroid receptor coactivator 1; CAT, chloramphenicol acetyltransferase.

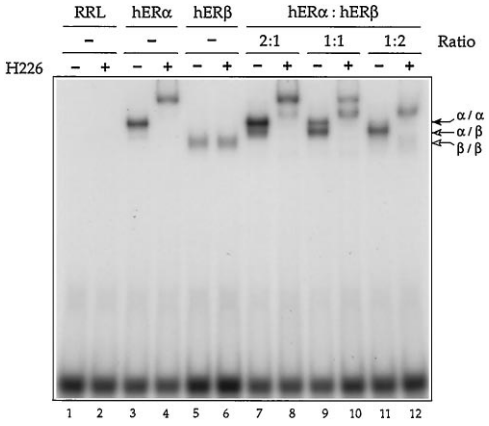

FIG. 1. **DNA binding activity of ERα, ERβ, and ERα/ERβ heterodimers.** Equivalent amounts of *in vitro* translated ERα, ERβ, or different ratios of the two, as indicated, were incubated with a ^{32}P-labeled ERE in the presence or absence of ER antibody H226. As controls, the unprogrammed lysate was analyzed (*tracks 1* and *2*). The position of the α/α homodimer, α/β heterodimer, and β/β homodimer is shown on the *right-hand side*.

samples were applied directly onto prerun nondenaturing 7% polyacrylamide gels (17), and complexes were detected by autoradiography or scanning with a PhosphorImager (Molecular Dynamics).

RESULTS

The DNA binding activity of ERα and ERβ was tested using *in vitro* translated receptors and a consensus estrogen response element in a gel shift assay. Both ERα and ERβ bound to the element, and the mobility of ERα, but not that of ERβ, was retarded in the presence of the hERα antibody, H226 (Fig. 1). When the two receptors were cotranslated we were able to detect a complex with an intermediate mobility corresponding to ERα/ERβ heterodimers, in addition to ERα homodimers (Fig. 1, *tracks 7–12*). Their mobility was retarded by H226 consistent with the presence of ERα in both complexes. Their relative amounts varied depending on the input ratio of the two receptors but it is noteworthy that ERβ homodimers were barely detected even when ERβ was expressed in 2-fold excess over ERα (Fig. 1, *track 11*). Their affinity for DNA was then determined by carrying out gel shift experiments over a wide range of probe concentrations (Fig. 2). We found that ERα homodimers and ERα/ERβ heterodimers bound to DNA with a similar K_d of approximately 2 nM whereas that of ERβ homodimers was about 4-fold greater.

We next analyzed the DNA binding activity of ERα and ERβ when they were expressed in COS-1 cells. When ERα alone was expressed, we observed two complexes, a major upper complex, corresponding to the ERα homodimer, and an additional complex that is probably generated by proteolysis. It seems to lack N-terminal sequences since it is recognized by a monoclonal antibody specific for the C-terminal F region (21) (data not shown). Similar results have been reported previously (22). As expected, the mobility of ERα but not ERβ was retarded in the presence of the specific hERα antibody, H226. When equivalent amounts of ERα and ERβ expression vector were coexpressed, heterodimers were the predominant form observed, and ERβ homodimers were not detected (Fig. 3*A*). We then used these extracts to compare the effect of 17β-estradiol and 4-hydroxytamoxifen on the DNA binding activity of the three dimeric forms (Fig. 3*B*). As previously demonstrated for ERα (7), the DNA binding activity of both ERβ and ERα/ERβ heterodimers was unaffected by ligand binding, but their mobilities were slightly increased in the presence of 17β-estradiol (Fig. 3*B*, *tracks 2*, *5*, and *8*). Thus, we conclude that ERα/ERβ heterodimers, expressed in intact cells, are capable of forming on DNA and that

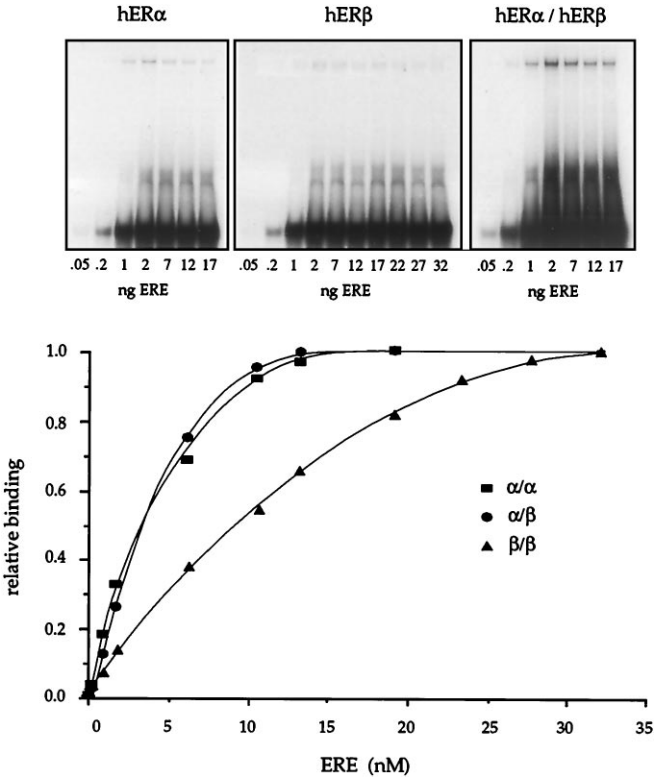

FIG. 2. **DNA binding affinity of ERα, ERβ, and ERα/ERβ heterodimers.** *In vitro* translated ERα, ERβ, or both (at a ratio of 2:1 in favor of ERβ) were analyzed for DNA binding activity in a gel shift assay over the range of DNA concentrations indicated. Radiolabeled probe alone was used up to a concentration of 6 nM (2 ng), and higher concentrations were achieved by diluting the labeled probe with nonlabeled oligonucleotide, and the resultant reduction in specific activity was taken into account in the calculations. Gels were then scanned with a PhosphorImager, and the relative binding of the different dimeric states was plotted against the concentration of ERE. Scatchard analysis was then used to determine the K_d for the different forms of receptor dimer.

ERα homodimers and ERα/ERβ heterodimers are preferentially formed.

Previous work with a series of ERα mutants has identified a region within the ligand binding domain of the estrogen receptor which is required for both receptor dimerization and high affinity DNA binding (15). We have used these mutants to determine whether the region required to form homodimers with a truncated version of ERα (mouse estrogen receptor (MOR)-182–599) is similar to that required to form heterodimers with ERβ on DNA. We find that the ability of R507A to form either ERα homodimers or ERα/ERβ heterodimers is markedly reduced (Fig. 4, compare *tracks 4* and *11*) while L511R and I518R, which show negligible homodimer formation, retain some ability to form ERα/ERβ heterodimers (Fig. 4, compare *tracks 6* with *13* and *7* with *14*). In contrast, mutation of Q510A had no affect on the dimerization of either receptor. A series of other mutations in this region of the receptor (A509R, L512V, L513G, I514R, L515G, L516A, H517A, R519G) was then screened to attempt to identify additional residues which could discriminate between homo- and heterodimerization, but all the mutants retained their DNA binding activity both as ERα homodimers and ERα/ERβ heterodimers (data not shown). We conclude that the region of ERα required for homodimerization overlaps that required for heterodimerization, but the two regions are not coincident.

We next assessed the transcriptional activity of ERα/ERβ heterodimers in transiently transfected COS-1 cells using the pEREBLCAT reporter gene. ERα and ERβ expression vectors

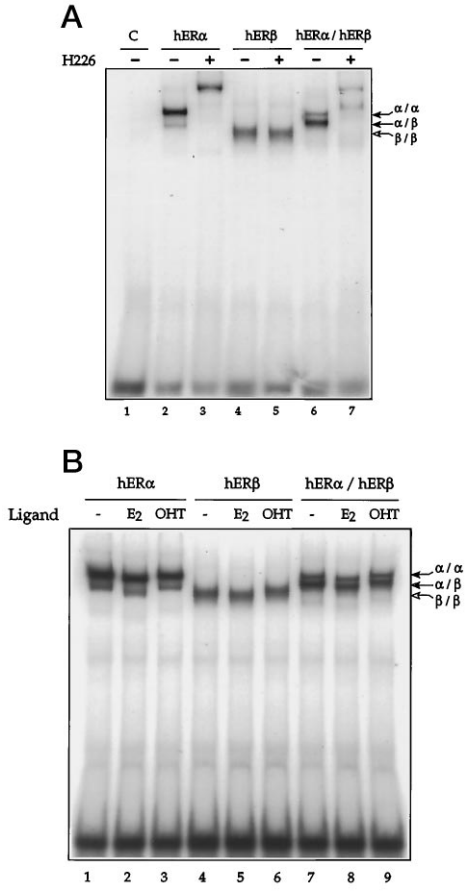

FIG. 3. **ERα and ERβ, expressed in COS-1 cells, bind as heterodimers to DNA.** Equal amounts of whole cell extracts, prepared from cells expressing ERα, ERβ, or both, were incubated with a ^{32}P-labeled ERE in the presence or absence of H226 antibody (*A*) or different ligands (*B*). 17β-Estradiol or 4-hydroxytamoxifen were tested at 2×10^{-8} M and 1×10^{-6} M, respectively. When ERα alone was expressed, we observed two complexes, a major upper complex, corresponding to the ERα homodimer, and an additional complex that is probably generated by proteolysis. It seems to lack N-terminal sequences since it is recognized by a monoclonal antibody specific for the C-terminal F region (21). The position of the α/α homodimer, α/β heterodimer, and β/β homodimer is shown on the *right-hand side*.

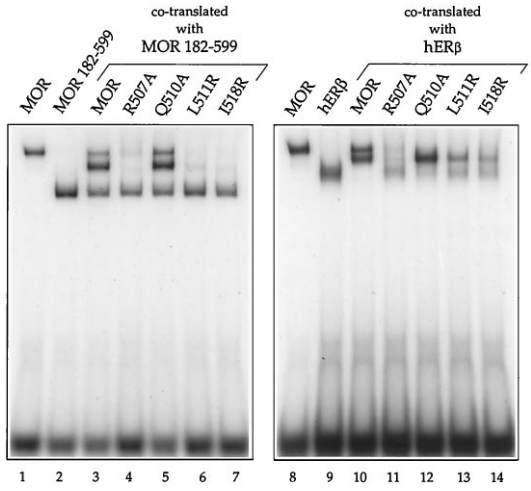

FIG. 4. **DNA binding activity of wild-type, mutant ERα, and ERβ.** The wild-type mouse ERα, MOR, and a series of mutants were cotranslated with either MOR-(182–599) (*tracks 3–7*) or ERβ (*tracks 10–14*) and tested for DNA binding activity using the gel shift assay. As controls, the wild type (*tracks 1* and *8*), truncated receptor MOR-(182–599) (*track 2*), and hERβ (*track 9*) were analyzed individually.

were tested individually or in combination at a ratio of 1:1 or 1:2 to minimize the relative amount of ER homodimers formed (see Fig. 3). The ability of ERα to stimulate transcription was slightly greater than that of ERβ (Fig. 5*A*), as previously reported for this reporter (4). Coexpression of ERα and ERβ resulted in an intermediate level of transcription that was blocked by the addition of the antiestrogens, 4-hydroxytamoxifen and ICI 182780. Similar results were obtained in HeLa cells (Fig. 5*B*). Therefore, since the heterodimer is the major dimeric form of the receptor under these conditions, it appears to retain its ability to stimulate transcription.

To obtain additional evidence to support our suggestion that ERα/ERβ heterodimers are capable of stimulating transcription we tested whether they were able to bind the coactivator, SRC-1, as previously demonstrated for ERα (10) and ERβ homodimers (4). This was achieved by analyzing the ability of the receptors, bound to DNA, to interact with a fragment of SRC-1, residues 570–780, that binds nuclear receptors in a ligand-dependent manner.[2] As shown in Fig. 6, when ERα, ERβ, or both were incubated with increasing amounts of GST-SRC-(570–780) in the presence and absence of ligand we could detect additional complexes in the gel shift assay. The interaction of SRC-(570–780) with ERα was dependent on the presence of ligand (Fig. 6, compare *tracks 7* and *8*) whereas there was an appreciable interaction with ERβ in the absence of ligand (Fig. 6, compare *tracks 13* and *14*). The interaction of SRC-1 with the heterodimer was enhanced in the presence of ligand (Fig. 6, compare *tracks 19* and *20*). As a control, we showed that these retarded complexes were not due to the binding of SRC-1 directly to an ERE (Fig. 6, *tracks 1* and *2*) but required the presence of receptor. Thus ERα/ERβ heterodimers, bound to DNA, are capable of recruiting SRC-1.

DISCUSSION

The main conclusion from our study is that human ERα and ERβ are capable of forming functional heterodimers on DNA. The relative distribution of ER homodimers and heterodimers will, at least in part, be dependent on the relative expression of the two receptors which varies widely in different cell types. Both ERα and ERβ have been detected in many tissues by reverse transcription-PCR or *in situ* hybridization (3, 23) but the relative amounts of receptor protein in specific cell types have yet to be determined. Nevertheless this preliminary analysis suggests that the expression of ERα may be greater than that of ERβ in epididymis, testis, pituitary ovary, uterus, adrenals, and heart. Given that ERα homodimers and ERα/ERβ heterodimers are preferentially formed over ERβ homodimers it seems that heterodimers are more likely to be formed than ERβ homodimers in these tissues. On the other hand, ERβ is expressed at higher levels in prostate, bladder, lung, thymus, and certain hypothalamic cells (3, 23), and so ERβ homodimers may be formed in these tissues.

The molecular basis for the reduced DNA binding activity of ERβ compared with that of ERα and ERα/ERβ heterodimers is unclear, but recent work indicates that the mouse ERβ also binds to an ERE less strongly than ERα (4). Differences in the DNA binding domains of the two receptors are unlikely to account for the variation since they differ by only two residues (1, 2), neither of which seems to be in a position that is likely to affect its DNA binding properties (24). An alternative possibility is that the receptors differ in their ability to dimerize. The major dimer interface in ERα has been mapped to a region of the hormone binding domain (15, 25) which is conserved and likely to correspond to helix 10 in nuclear receptors (26). The

[2] E. Kalkhoven, data not shown.

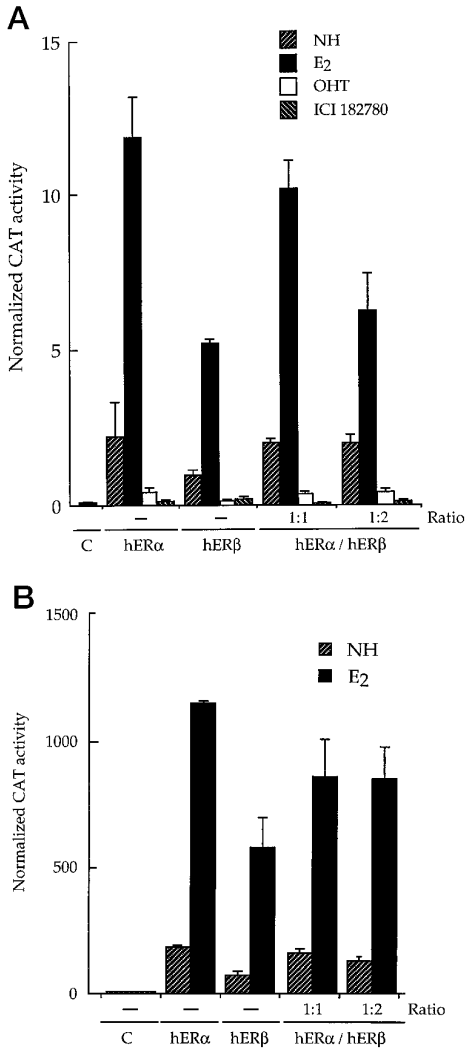

FIG. 5. **Transcriptional activity of coexpressed ERα and ERβ.** The ability of ERα and ERβ, expressed individually or in combination, to stimulate transcription of a reporter gene pEREBLCAT was tested by transient transfection in COS-1 (*A*) and HeLa cells (*B*). Transcriptional activity was determined in the absence or presence of 1×10^{-8} M 17β-estradiol (E_2), 1×10^{-7} M 4-hydroxytamoxifen, and 1×10^{-7} M ICI 182780 and corrected for the activity of the internal control pJ7lacZ. Activity of the reporter was also analyzed in the absence of transfected ER as a control (*C*). The *error bars* represent standard errors determined from two transfection experiments each carried out in duplicate.

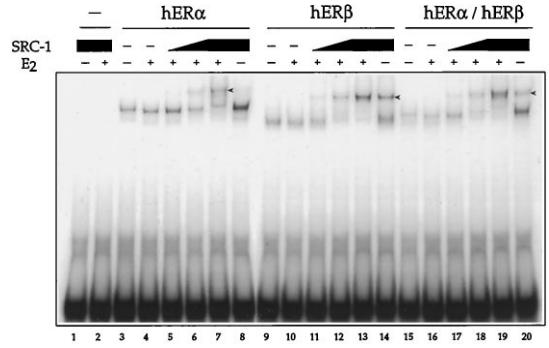

FIG. 6. **SRC-1 binds to ERα/ERβ heterodimers bound to DNA.** Equal amounts of ERα, ERβ, and ERα/ERβ heterodimers were incubated with increasing amounts of GST-SRC-(570–780) in the presence or absence of 2×10^{-8} M 17β-estradiol. As controls, GST-SRC-(570–780) was tested for its DNA binding activity in the absence of receptor. The position of receptors complexed with GST-SRC is indicated by *solid arrowheads*.

crystal structure of the ligand binding domain of RXR also indicates that the dimer interface comprises this helix and, to a lesser extent, helix 9 and the loop between helix 7 and 8 (27). Comparison of the sequence of the corresponding region in ERα and ERβ indicates that helix 10 is similar (13/18 residues are identical) but the loop region and helix 9 are poorly conserved in the two receptors. Thus, it seems likely that the residues required to form the dimer interface in ERα and ERβ homodimers are distinct. Although the precise dimerization interfaces in these receptors have yet to be identified, functional analysis of a series of mutations in helix 10 of ERα indicates that the residues required for the formation of ERα homodimers are similar but not identical to those required for ERα/ERβ heterodimerization.

Both ERα and ERβ are capable of stimulating the transcription of reporter genes in transfected cells and the activation functions, AF-1 and AF-2 characterized in ERα (5, 6, 8, 28), appear to be conserved in ERβ (4). When ERα and ERβ are expressed, under conditions when heterodimers are the predominant dimeric species, transcription of an ERE reporter gene is stimulated to an intermediate level compared with that of either homodimer suggesting that ERα/ERβ heterodimers are transcriptionally active. This is supported by our observation that ERα/ERβ heterodimers are capable of binding the coactivator SRC-1 (9, 29). We found that SRC-1 interacts with all three dimeric states of ER bound to DNA although its interaction with heterodimers was less dependent on ligand than that observed with ERα homodimers. The interaction of ERβ homodimers with SRC-1 was dependent on ligand in solution (4)[3] but not on DNA and, consistent with this, SRC-1 was found to augment the transcriptional activity of ERβ in the absence of ligand (4). Finally, the ability of the heterodimer to stimulate transcription was blocked by the antiestrogens, 4-hydroxytamoxifen and ICI 182780, as previously demonstrated for ER homodimers (4, 7, 8, 30).

The discovery of a second estrogen receptor raises many questions, most notably relating to their respective functions. The ability of ERα and ERβ to form heterodimers suggests that estrogen receptor may function in different dimeric states, and it is possible that they could be activated by selective ligands. In view of the similarity of their DNA binding domains it is doubtful whether different forms bind to distinct response elements, but they could activate different genes in different target cells given their distinct expression patterns.

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