Biochemical Pharmacology, Vol. 57, pp. 597–601, 1999.

ISSN 0006-2952/99/$–see front matter
PII S006-2952(98)00355-4

RAPID COMMUNICATION

Interaction of Human Estrogen Receptors α and β with the Same Naturally Occurring Estrogen Response Elements

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ABSTRACT. Estrogen receptors are derived from two different gene products referred to as estrogen receptor-α (ER-α) and ER-β. Both receptors bind to the consensus estrogen response element (ERE) present in the vitellogenin gene, but their binding to hormone response elements present in other estrogen responsive genes has not been reported yet. Using *in vitro* expressed human receptors, we now show that ER-β binds to a panel of six endogenous hormone response elements (vitellogenin, c-*fos*, c-*jun*, pS2, cathepsin D, and choline acetyltransferase) already known to bind ER-α and confer estrogen inducibility to reporter constructs. Binding of ER-α and ER-β occurred at similar DNA concentrations for some EREs, but different DNA concentrations were required to form complexes of the two receptors with other elements. These results illustrate for the first time by direct receptor–DNA binding studies that both ER-α and ER-β bind to a number of EREs present in endogenous hormone regulated genes, and further suggest that the two forms of the receptor display different patterns of affinities for naturally occurring hormone response elements. BIOCHEM PHARMACOL **57**;6:597–601, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. estrogen receptor-α; estrogen receptor-β; estrogen response elements; DNA binding

A second form of the ER†, termed ER-β to distinguish it from the classic ER or ER-α, has been cloned recently [1–3]. ER-α and ER-β are arranged into similar domains, although the degree of homology varies widely among regions, being highest in the DNA binding domain and considerably lower in others [4]. Both receptors (a) exhibit the same affinity for the endogenous estrogen 17β-estradiol [5], (b) are inhibited by pure antiestrogens [3], (c) can activate transcription from the vit-ERE, which is commonly referred to as the consensus ERE [3, 6], and (d) can bind to the vit-ERE sequence as either homo- or heterodimers [7, 8]. The two receptors display generally similar ligand binding profiles, although they interact differentially with phytoestrogens such as genistein and coumestrol [5].

While many of the biochemical properties of the two receptors are similar, they differ substantially in their tissue distribution, which raises questions about potential differences in the biologic function and tissue-selective actions of the two receptors. For example, they might be adapted to regulate the expression of genes that are highly expressed in tissues where one form predominates, and/or the two receptors might selectively regulate the expression of different genes in tissues where both forms are expressed. To understand the biology of this family of receptors, it thus is important to evaluate their interactions with the regulatory sequences present in a variety of target genes. Both receptors bind to the consensus ERE (5′-GGTCAnnnTGACC-3′), but this sequence ONLY mediates the expression of a single endogenous gene (i.e. vitellogenin). All other EREs identified to date have one or more base changes from the consensus sequence—the most common motif being one half-site identical to that in the vit-ERE and a second half-site with considerable variability in nucleotide sequence [9]. However, to date there are no reports of the binding of ER-β to any other regulatory sequences involved in estrogen action. This prompted us to investigate the binding of ER-α and ER-β to a series of EREs present in genes regulated by estrogens in intact cells to determine if the two receptors demonstrate significant differences in their patterns of binding to naturally occurring regulatory elements.

MATERIALS AND METHODS

Materials

Enzymes were obtained from Boehringer Mannheim, and oligonucleotides were synthesized by Genosys or National Biosciences. Recombinant human ER-α (66 kDa) and ER-β (53 kDa) [2] were obtained from Panvera. Polyacrylamide gel electrophoresis supplies were obtained from

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† *Abbreviations*: ER, estrogen receptor; ERE, estrogen response element; vit, vitellogenin; and PRE, progesterone response element.

TABLE 1. Oligonucleotide sequences used in gel-shift experiments

vit-ERE	5′-GATCCGTCAGGTCACAGTGACCTGATG-3′
fos-ERE	5′-TCGACCTTTATCCAGGTCACCACAGCCCAGGCCATG-3′
mt-*fos*-ERE	5′-TCGACCTTTATCCATTTCACCACAGCCCAGGCCATG-3′
jun-ERE	5′-GATCCTGAAGCAGAGCATGACCTTGAACTGAAGCAGAGCATGACCTTGAA-3′
pS2-ERE	5′-GATCTGCAAGGTCACGGTGGCCACCCC-3′
Cath D-ERE	5′-GATCAGCTGGGCCGGGCTGACCCCGCG-3′
ChAct3	5′-GATCCAGGAGGCCACGATGACATGCTC-3′
PRE	5′-GATCCAGAACAAACTGTTCTAGCTACG-3′

The sequences (sense strand) EREs are underlined. Abbreviations: vit, vitellogenin; mt, mutant; cath D, cathepsin D; and ChAct3, choline acetyltransferase. The *jun* ERE was present as two copies tandemly attached.

Bio-Rad. Antibody H-222 was provided by Dr. Geoffrey Greene of the University of Chicago. All other materials used were of the highest grade commercially available.

Oligonucleotide Preparation and Gel-Shift Assays

The nucleotides containing the natural EREs (underlined) used are shown in Table 1. These include EREs from the vitellogenin gene [6], pS2 [10], cathepsin D [11], choline acetyltransferase [12], c-*fos* [13], and c-*jun* [14]. Oligonucleotides were labeled to a specific activity of 0.5 to 1.0 $\times$ 10^6 cpm/ng with T4 polynucleotide kinase, and gel-shift assays were done as previously described [15] with minor modifications. Briefly, 12.5 to 100 ng of the receptor protein (as indicated for each experiment) was incubated with the polynucleotide dI–dC (1 μg/5 μg receptor) in 20 μL of TND buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM dithiothreitol) containing 1 mg/mL of BSA at 4°. ERs were diluted just before use to the desired concentration (100 ng/μL for studies in Figs. 2–4 and 25 ng/μL for those in Fig. 5) with the storage buffer containing 1 mg/mL of BSA as described for ER-α in the manufacturer's brochure. After 20 min on ice, 0.1 to 1.0 ng of a ^{32}P-labeled oligonucleotide containing an ERE sequence was added and incubated for 20 min at 4°. The entire mixture was then loaded onto 4–8% polyacrylamide gels (30:1 acrylamide: bisacrylamide) and run at 160 V for 90 min. When ER antibody was used, it was added to the reaction mixture containing ER-α and incubated for 1 hr before adding the probe. The position of the ER–DNA complexes is illustrated by the arrowheads in the figures. All experiments were performed at least twice, and a representative gel-shift is shown for each figure.

RESULTS

As an initial positive control, we tested the ability of the receptors to bind specifically to the vit-ERE as previously reported [8, 16], and to determine the usable range of ER protein concentration. As shown in Fig. 1, both ER-α and ER-β bound the vit-ERE at protein levels between 25 and 100 ng under our assay conditions. Binding of both receptors was specific as indicated by competition with a 100-fold excess of unlabeled DNA. Note also that the migration of the ER-β complex was slightly greater than that of ER-α. This was expected [8], since the ER-β has a slightly lower molecular weight [4].

We next tested the ability of the two receptors to bind to the ERE we previously identified in the 3′-untranslated region of the murine c-*fos* gene [13]. This sequence contains one half-site identical to the vit-ERE (GGTCA) but a second half-site that differs in three of five positions (CAGCC) (see Table 1). We have demonstrated previously that this element confers estrogen inducibility on both homologous and heterologous promoters [13] and binds both the human [13] and rat [15] ER-α. We also tested the ability of both receptors to bind to an element containing a mutated c-*fos* 5′-half-site (TTTCA) shown in Table 1, since these base changes destroy both hormone inducibility and ER-α binding [13]. Both ER-α and ER-β bound to the native *fos* element, and this binding was competed by an excess of unlabeled oligodeoxynucleotide (Fig. 2). In contrast, neither receptor bound to the mutated element.

We further tested the DNA binding specificity of ER-α and ER-β by examining binding to another steroid hormone response element. As seen in Fig. 3, neither receptor bound the PRE. As other controls, we demonstrated that

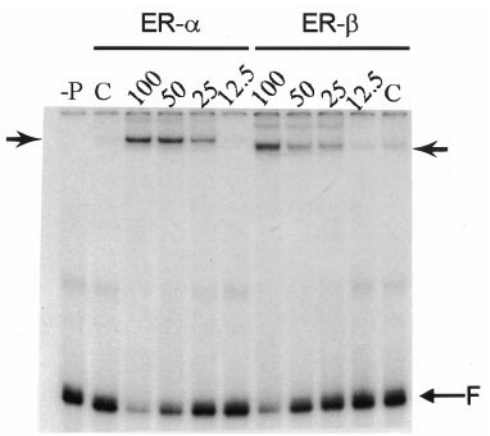

FIG. 1. Interaction of ER-α and ER-β with the vit-ERE. The indicated amounts of ER protein (in ng) were incubated with the labeled vit-ERE, and electrophoresed (8% gel) as described in Materials and Methods. Key: P, probe alone without ER; C, 100-fold excess unlabeled vit-ERE; and F, free probe. This experiment was performed at least twice, and a representative gel-shift is shown.

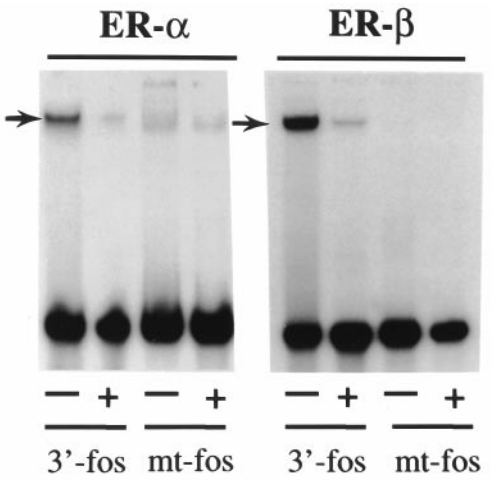

FIG. 2. Binding of ER-α and -β to the endogenous c-*fos* ERE. ERs were incubated with the labeled 3′-*fos* ERE (3′-*fos*) or an inactive mutated sequence (mt-*fos*) plus (+) or minus (−) a 100-fold excess of the corresponding non-labeled DNA. Electrophoresis (6% gel) was performed as described in Materials and Methods. This experiment was performed at least twice, and a representative gel-shift is shown.

the *c-fos*–ER-α complex was supershifted by an antibody raised against this receptor, and that heat-inactivated ER-α did not form a DNA complex (Fig. 3). Antibodies for ER-β supershifts are not yet available, but the binding of this receptor to the vitellogenin (Fig. 1) and *fos* (Fig. 2) EREs was clearly specific since: (a) it was competed by an excess of both these sequences, (b) it did not bind to the mutated *fos* element (Fig. 2), or (c) it did not bind to another hormone response element (Fig. 3).

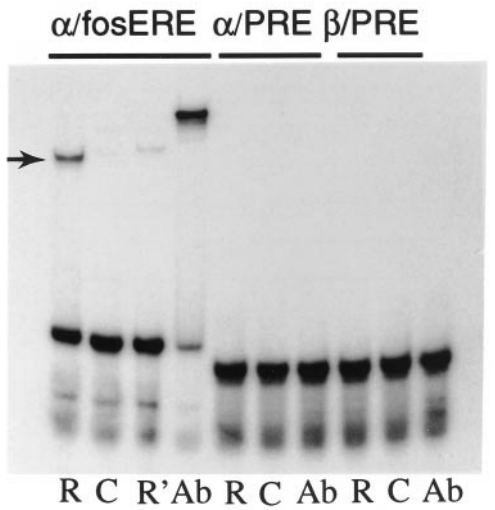

FIG. 3. Specificity of ER-α and -β binding. ERs were incubated with the labeled c-*fos* ERE or PRE, and electrophoresis was performed (5% gel) as described in Materials and Methods. Key: R, receptor + labeled probe; C, competition with 100-fold excess unlabeled DNA; R', incubation with heat-inactivated receptor (37°, 1 hr); and Ab, incubation plus antibody H-222 raised against ER-α. This experiment was performed at least twice, and a representative gel-shift is shown.

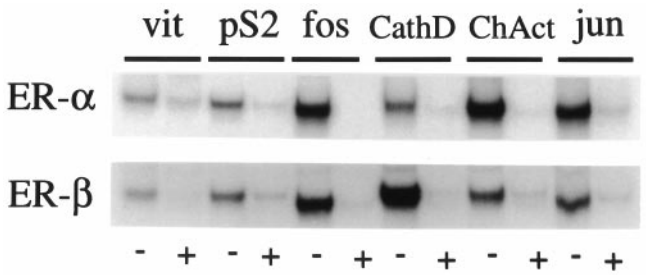

FIG. 4. ER-α and -β binding to natural EREs. ER-α (top panel) or -β (bottom panel) was incubated with labeled DNA containing the EREs depicted in Table 1 in the presence (+) or absence (−) of a 100-fold excess of the unlabeled sequence. Electrophoresis was performed in a 5% non-denaturing gel as described in Materials and Methods. This experiment was performed at least twice, and a representative gel-shift is shown.

Since both ERs bound to an endogenous non-palindromic ERE as well as the vit-ERE, we next investigated their binding to a panel of endogenous elements including pS2 [10], cathepsin D [11], choline acetyltransferase [12], and c-*jun* [14]. Both ER-α (Fig. 4, top panel) and ER-β (Fig. 4, bottom panel) bound all four elements in addition to the c-*fos* and vit-EREs, as previously seen. Binding was specific as judged by competition with an excess of unlabeled DNA in each case. The difference in intensity of the bands formed in Fig. 4 with the various elements does not necessarily imply differences in binding affinities or amounts of receptor bound, since the various oligos were end-labeled in separate reactions and thus have different specific activities. Also, based on previous gel-shift studies utilizing ER-α and several of the elements used in this study [13–15], we used 0.1 to 0.2 ng of the vit- and pS2-EREs, but 1 ng of DNA for the other four probes. It is interesting to note in Fig. 4 that there were slight differences in the migration of ER complexes with different EREs, and these differences were observed consistently in other studies performed during the course of this work. We are uncertain at present whether this is due to a differential bending of the various DNA sequences induced by receptor binding [17] or to other differences.

In a final experiment we examined the concentration dependence of several elements (c-*fos*, cathepsin D, and choline acetyltransferase) for complex formation with the two receptors. In this study a fixed concentration (25 ng/μL) of either ER-α or -β was incubated with increasing concentrations of the labeled oligodeoxynucleotides containing the three EREs (see Table 1) prior to electrophoresis. As seen in Fig. 5, both receptors showed a similar pattern of complex formation with the c-*fos* ERE. In contrast, ER-β required somewhat higher DNA concentrations than ER-α to form complexes with the choline acetyltransferase ERE, and the β receptor required a substantially higher DNA concentration than the α receptor to form complexes with the cathepsin D element.

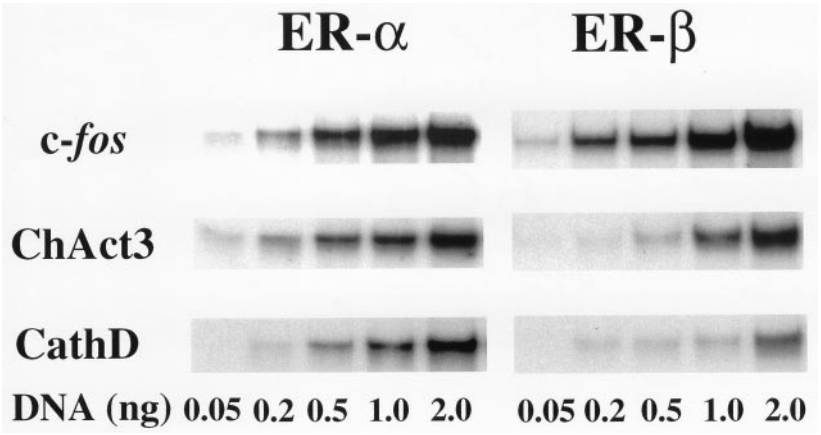

FIG. 5. DNA dependence of ER-α and ER-β binding to different EREs. The receptors (25 ng per reaction mixture) were incubated with various concentrations of radiolabeled oligonucleotides containing c-*fos*, choline acetyltransferase, or cathepsin D (see Table 1) as indicated. Electrophoresis was then performed in a 6% non-denaturing gel as described in Materials and Methods. This experiment was performed at least twice, and a representative gel-shift is shown.

DISCUSSION

We have examined the binding of ER-α and ER-β to a variety of naturally occurring EREs including: (a) the palindromic vitellogenin element, (b) four elements (*fos*, *jun*, pS2, and cathepsin D) of the type most frequently observed in endogenous hormone-responsive genes, i.e. one perfect half-site and a second half-site with 1–3 base changes from the palindromic vit-ERE, and (c) a non-palindromic element (choline acetyltransferase) with overall similarity to the vit-ERE (eight of ten nucleotides identical) but without a perfect half-site. Our results indicate that both ER-α and ER-β bind to all six elements. This binding is specific, since neither receptor bound to the PRE or a mutated c-*fos* sequence that lacks biologic activity [13] and receptor binding [15, and this work]. These findings thus are consistent with many published reports showing that high affinity binding of nuclear receptors to DNA is very sequence-specific, and indicate that the binding of both ER-α and ER-β is specific for sequences with some homology to the vit-ERE.

While our findings demonstrate that both estrogen receptors can bind to all the naturally occurring response elements tested, the two receptors form ERE complexes at similar DNA concentrations in some cases (e.g. c-*fos*), but at different DNA concentrations in others (e.g. choline acetyltransferase and cathepsin D). If one assumes that the stoichiometry of receptor binding to the various elements is similar, and that all the sequences bind to the same region of the receptor, this observation indicates different receptor affinities for the different DNA sequences. In a similar study, Cowley *et al.* [8] found a 4-fold difference in affinity of ER-α and -β to the vit-ERE. These results raise the possibility that the two forms of the receptor may produce different patterns of gene activation *in vivo*. It is important to note, however, that intact cells contain chromosomal proteins, other regulatory proteins, and mixtures of receptor homo- and heterodimers that could affect the relative binding of the two receptors to the regulatory elements present in different genes. In this regard, our DNA binding studies were done with receptor concentrations of approximately 20–80 nM (25–100 ng protein/20 μL reaction volume), and it has been estimated that the concentration of ER-α in a typical target organ cell such as the uterus is approximately 30 nM [18]. Given the numerous uncertainties and assumptions present in such estimates, these values are nevertheless consistent with *in vivo* interactions between ER-α and ER-β and the EREs we have studied.

It is clear that ER-α and ER-β have different tissue distributions, which could lead to different biologic roles, but a major issue is whether the two receptors have differences in their biochemical functions that could contribute to different physiological and/or pharmacological actions. Others have shown that both receptors show generally similar ligand binding profiles for endogenous estrogens and most estrogenic drugs. Both receptors can activate transcription from the consensus ERE, but Cowley *et al.* [8] and Tremblay *et al.* [3] noted differences in the affinities of the two proteins for that element. Our results now indicate that both receptors can bind to all the naturally occurring EREs we have tested, but may have different patterns of affinity for these endogenous hormone response elements. This raises the possibility that these differing affinities for regulatory DNA sequences could contribute to different patterns of gene activation by the two receptors, but this remains to be proven or disproven by further studies on transcriptional activation from different hormone response elements.

This work was supported by NIH Grant HD-08615. We would like to thank Ms. Heidi Porter for editorial assistance.

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