Human Estrogen Receptor β Binds DNA in a Manner Similar to and Dimerizes with Estrogen Receptor α

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The cloning of a novel estrogen receptor β (denoted ERβ) has recently been described (Kuiper, G. G. J. M., Enmark, E., Pelto-Huikkko, M., Nilsson, S., and Gustafsson, J-A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5925–5930 and Mosselman, S., Polman, J., and Dijkema, R. (1996) FEBS Lett. 392, 49–53). ERβ is highly homologous to the “classical” estrogen receptor α (here referred to as ERα), has been shown to bind estrogens with an affinity similar to that of ERα, and activates expression of reporter genes containing estrogen response elements in an estrogen-dependent manner. Here we describe functional studies comparing the DNA binding abilities of human ERα and β in gel shift assays. We show that DNA binding by ERα and β are similarly affected by elevated temperature in the absence of ligand or in the presence of 17β-estradiol and the partial estrogen agonist 4-hydroxy-tamoxifen. In the absence of ligand, DNA binding by ERα and β is rapidly lost at 37 °C, while in the presence of 17β-estradiol and 4-hydroxy-tamoxifen, the loss in DNA binding at elevated temperature is much more gradual. We show that the loss in DNA binding is not due to degradation of the receptor proteins. However, while the complete antagonist ICI 182,780 does not “protect” human ERα (hERα) from loss of DNA binding at elevated temperature in vitro, it does appear to protect human ERβ (hERβ), suggestive of differences in the way ICI 182,780 acts on hERα and β. We further report that ERα and β can dimerize with each other, the DNA binding domain of hERα being sufficient for dimerization with hERβ. Cell and promoter-specific transcription activation by ERα has been shown to be dependent on the differential action of the N- and C-terminal transcription activation functions AF-1 and AF-2, respectively. The existence of a second estrogen receptor gene and the dimerization of ERα and β add greater levels of complexity to transcription activation in response to estrogens.

The estrogen receptor α (ERα) is a member of a superfamily of transcription factors that induce transcription of target genes by binding to cis-acting enhancer elements in promoters of responsive genes (for reviews, see Refs. 3–10). Cloning of the cDNAs encoding the human estrogen receptor (hERα) and comparison with ER from other species have been used to divide ERα into six regions, A to F, on the basis of differing amino acid sequence homology (11). Functional studies have shown that region C encodes the DNA binding domain (DBD), and region E contains the hormone/ligand binding domain (LBD) (12–13). Furthermore, the ERα LBD has been shown to contain a hormone-inducible transcription activation function (AF-2) and the N-terminal A/B region (which is highly divergent and variable in length in ER from different species) also contains a transcription activation function (AF-1). AF-1 and AF-2 can activate transcription independently and synergistically act in a promoter- and cell-specific manner (12, 14–19). Region D is involved in binding to hsp90 (20), as well as containing nuclear localization signals (21), and plays a part in stabilizing DNA binding by the DBD (22). Region F appears to play a role in modulating transcriptional activation by ERα (23).

The mechanisms of anti-estrogen action have been extensively studied. In particular, anti-estrogens such as tamoxifen and ICI 164,384 (or its derivative ICI 182,780) antagonize the effects of estrogens by competing with estrogen for binding to the estrogen receptor. Tamoxifen, and its derivative 4-hydroxy-tamoxifen, show partial agonistic activity by inhibiting transcription activation by AF-2 but enabling transcription through AF-1 (18–19, 24). ICI 164,384, on the other hand, is a complete antagonist that does not prevent DNA binding by ER but inhibits transcription activation by both AF-1 and AF-2 (25). Thus, anti-estrogens do not appear to act by preventing DNA binding by ERα and may act by inactivating (or preventing the activation of) the transcription activation functions (see also Ref. 26). Metzger et al. (25) showed, however, that at elevated temperature (37 °C), hERα undergoes a reduction in its capacity to bind to an ERE in vitro. The reduction in DNA binding is much faster in the absence of ligand than in the presence of E2 or the partial agonist OHT. Interestingly, ICI 164,384 does not “protect” hERα from losing DNA binding capacity at 37 °C. In vivo ICI 164,384 binding reduces the half-life of ERα (27–29). This reduction in half-life may result (at least in part) from disruption of nucleocytoplasmic shuttling of ERα by ICI 164,384 or ICI 182,780 (30).

On the basis of DNA binding and dimerization properties, members of the nuclear receptor superfamily have been divided into four broad classes (for reviews, see Ref. 9 and references therein). Class I receptors include the steroid hormone receptors that bind to palindromic DNA elements (two half-sites arranged as inverted repeats) as homodimers. ERα binds as a homodimer to two half-sites with the sequence RGGTCA, separated by 3 bp: base pairs. Amino acids near the C terminus of the first zinc finger in the so-called P-box determine the half-life of protein.
site sequence to which the receptor binds while the amino acids in the D-box (near the N terminus of the second zinc finger) provide important protein-protein contacts in receptor dimers (see Refs. 31 and 32, and references therein). While sequences in the DDB are adequate for receptor dimerization, sequences within the LBD are required for dimerization of the full-length ERα (33).

Estrogens have long been understood to play important roles in regulating the development and in the maintenance of the female reproductive system, being involved in maintaining fertility and in regulating bone development and maintenance. The failure to detect mutations in the ERα gene has also been thought to imply that its mutation is lethal. Recent studies from Korach and co-workers (34) describing ERα knockout mice have shown that, while absence of the ERα gene is not lethal and female and male mice survive to adulthood, they are infertile with gross gonadal deformities and display decreased skeletal bone density. No estrogen sensitivity was detectable in mutant female mice using uterine growth assays nor was induction of the estrogen-responsive lactoferrin gene expression seen. Mammary gland development in female mice was rudimentary and expression of the progesterone receptor gene was not increased in response to estrogens. The results of these studies have provided clear evidence for the importance of ERα in the development and maintenance of the female but also of the male reproductive systems, as well as for bone development and maintenance. There has been little evidence from these studies, however, for the involvement of ERα in prenatal development of the reproductive tract, and interestingly, some estrogen binding activity (5% compared with wild-type mice) was detectable in the uteri of mutant females (34–37).

Somewhat surprisingly, the cloning of a second estrogen receptor gene (ERβ) has recently been reported in the rat (1) and in man (2). ERβ is highly homologous to ERα, displaying 96 and 58% homology (when hERα and β are compared) in the DNA and hormone binding domains, respectively. The rat ERβ is most highly expressed in the prostate and ovary (1), while hERβ and hERβ are most highly expressed in the prostate and ovary (1), while the amino acid sequence of hERβ differs at 37°C. We further show that ERβ and β interact differently at 37°C. We further show that ERα and β can heterodimerize. The implications of these findings are discussed. In this study we wanted to examine in vitro DNA binding by human ERβ and analyze the effects of estrogen and the antiestrogens 4-hydroxy-tamoxifen and ICI 182,780 (ICI) on DNA binding. We have compared in vitro DNA binding by hERα and β. Our results indicate that the complete antagonist ICI may act on hERα and β differently at 37°C. We further show that ERα and β can heterodimerize. The implications of these findings are discussed.
and incubation at 25 °C for 15 min (incubation). Different preincubation and incubation times and temperatures were also used, as described in the text and figure legends.

Receptor-DNA complexes were separated on 5% polyacrylamide gels (30% acrylamide, 1% bis-acrylamide, containing 0.5 × Tris-borate-EDTA). Gels were electrophoresed in 0.5 × Tris-borate-EDTA at 150 V and dried before autoradiography. The bands corresponding to the specific retarded complex of hER-ERE and to unbound ERE were quantified using a Bio-Rad molecular imager.

Immunoblot Analysis—WCE were fractionated using 10% SDS-polyacrylamide gel electrophoresis, and immunodetection was performed using a monoclonal antibody B10 directed against the region B of hER (41) except that immunodetection was carried out by incubation with alkaline phosphatase-labeled goat anti-rabbit IgG and revelation with BCIP/NTB (Promega). For detection of hERβ1, blots were incubated with biotinylated M2 monoclonal antibody (IBI) at 1 mg/ml for 2 h at 25 °C, followed by 3 × 15 min washes using 0.05% Tween-20PBS. The immunoblots were then incubated with 1 mg/ml avidin–alkaline phosphatase conjugate for 2 h at 25 °C, followed by further washing and revelation using BCIP/NTB.

RESULTS

PCR Amplification and Cloning of Human Estrogen Receptor β—To examine the DNA binding properties of hERβ, oligonucleotides were designed to PCR amplify overlapping 5′ and 3′ portions of the hERβ open reading frame using the reported cDNA sequence (2). The oligonucleotide primers were designed to contain restriction sites to facilitate cloning, and the PCR products were cloned into the mammalian expression vector pSG5, which also contains the T7 promoter enabling in vitro transcription (39), as detailed under “Materials and Methods.” Base pairs 7–1485 of hERβ (2) were cloned into pSG5 to give hERβ0 (Fig. 1A). An oligonucleotide linker encoding amino acids comprising the “FLAG” epitope was ligated N-terminal to the predicted hERβ translational start methionine to give hERβ1 (Fig. 1A) for immunodetection using a commercially available monoclonal antibody M2. Automated sequencing was used to confirm the sequences of hERβ0 and hERβ1.

In in vitro transcription/translation of hERβ0 and hERβ1 was performed to determine the size of the proteins. In in vitro transcription/translation of pSG5 and HEG0 (pSG5 containing the hERα open reading frame (38)) were used to provide negative controls, respectively. Fig. 1B shows the results of in vitro transcription using [35S]methionine in a rabbit reticulocyte lysate and fractionation on 10% SDS-PAGE, followed by autoradiography. In vitro translation of HEG0 gave a band at the expected size of 67 kDa (Fig. 1B, lane 2) while in vitro translation of hERβ0 resulted in a polypeptide of about 54 kDa, in agreement with the size predicted from examination of the hERβ cDNA sequence (2). A product of 55 kDa was observed for hERβ1, slightly larger than hERβ0, as would be expected from the presence of an additional 13 amino acids N-terminal to the translation initiation codon of hERβ0.

Binding of hERβ to an ERE in Vitro—CO-1 cells, maintained in DMEM containing 5% FCS, were transfected with pSG5, HEG0, or hERβ0, and WCE were prepared as described under “Materials and Methods.” Extracts were first preincubated in gel shift buffer (containing KCl to a final concentration of 80 mM) at 4 °C for 15 min in the presence of 2.0 μg of poly(dI-dC). Radiolabeled ERE or a mutant ERE was then added to the reaction for 15 min at 25 °C before running on a 5% nondenaturing polyacrylamide gel (Fig. 2). Specific DNA-receptor complexes were observed in both HEG0 and hERβ0 when incubated with the wild-type (ERE) but not the EREM (Fig. 2A, compare lanes 1–3 with lanes 4–6), demonstrating specific binding of hERs α and β to ERs.

Next we examined hERβ/ERE complex formation in the presence or absence of ligands. COS-1 cells, maintained in DMEM without phenol red and containing 5% charcoal-stripped FCS, were transfected with pSG5, HEG0, or hERβ0. Ligands (17β-

estradiol (E2), 4-hydroxy-tamoxifen (OHT) or ICI 182,780 (ICI)) were added to 100 nM 2 h prior to harvesting. Gel shift assays were performed as above. Specific complex formation was observed in each case (Fig. 2B). Similar amounts of complex were observed in the presence of E2 or OHT (HEG0, lanes 3 and 4; hERβ0, lanes 6 and 7), whereas in the absence of ligand (lane 2) or in the presence of ICI (lane 5), lower amounts of complex were observed for HEG0 as has previously been described (see the Introduction). Similarly, in the presence of ICI, lower levels of complex were observed for hERβ0 (lane 9).

In addition, it was clear that the HEG0-ERE and the hERβ0/ERE complexes migrated faster in the presence of E2 than in the absence of ligand or the presence of OHT or ICI.

Effect of Temperature on in Vitro ERE Binding by hERβ—The experiments described above showed that DNA binding by hERs and hERβ was differentially affected by the different ligands. Mauger et al. (24) have shown that preincubation and/or incubation at 37 °C drastically reduced ERE binding by HEG0. To examine the effect of temperature on DNA binding by hERβ, we performed gel shift assays in which HEG0 and hERβ were preincubated at 4 or 37 °C in the presence or absence of ligands. hERβ1 was used to perform immunoblotting to determine whether differences in DNA binding were due to degradation of the receptor in the gel shift assay. High
Absence of ligand similarly affected DNA binding by hERΔβ0. Results are in agreement with those previously described containing 5% FCS, using 32P-labeled ERE (lanes 1–3) or mutant ERE (EREM, lanes 4–6). The extracts were preincubated at 4 °C for 15 min before addition of ERE or EREM and incubation at 25 °C for 15 min before running on a 5% nondenaturing polyacrylamide gel. B, gel shifts were performed as in panel A using cell extracts prepared from COS-1 cells transiently transfected with pSG5 (lanes 1), HEG0 (lanes 2–5), or hERΔβ0 (lanes 6–9) and incubated with the radiolabeled ERE. The cells were cultured in DMEM, without phenol red, containing 5% charcoal-stripped FCS. Ligands were added to the transfectants two h before harvesting, either vehicle alone (lanes 1, 2, and 6) or 100 nM E2 (lanes 3 and 7), OHT (lanes 4 and 8), or ICI (lanes 5 and 9).

Salt extracts of COS-1 cells transfected with HEG0 or hERΔβ1 were preincubated at 4 °C or 37 °C for 30 min in the presence or absence of ligands, followed by addition of labeled ERE and a further incubation at 37 °C for 30 min. In the presence of E2 or OHT, preincubation at 4 or 37 °C had little effect on DNA binding by HEG0 (Fig. 3A, compare lanes 3 and 4 and lanes 5 and 6), whereas in the absence of ligand or in the presence of ICI, preincubation at 37 °C resulted in greatly reduced DNA binding (compare lanes 1 and 2 and lanes 7 and 8). These results are in agreement with those previously described (25). Absence of ligand similarly affected DNA binding by hERΔβ1, while DNA binding in the presence of E2 or OHT was unaffected (Fig. 3A, lanes 9–16). Some reduction in DNA binding was also evident when ICI was used, although the effect was less marked compared with HEG0. Western blotting using monoclonal antibodies B10 and M2 for detection of HEG0 and hERΔβ1, respectively, showed that the reduced DNA binding was not due to degradation of HEG0 or hERΔβ1 during incubation (Fig. 3B).

The above results indicate that hERα and β are inactivated in some way by preincubation at 37 °C in the absence of ligand or in the presence of ICI and offers further evidence to indicate that hERβ binds DNA in a manner similar to hERα in the absence of ligand and in the presence of E2 or the anti-estrogens OHT but suggest that DNA binding by hERα and β is affected differently by ICI. To more fully examine the effect of temperature on DNA binding by hERβ and compare the DNA binding properties of hERα and β, we performed further gel shift experiments in which untreated or ligand-treated HEG0- and hERΔβ0-containing COS-1 cell extracts were preincubated for varying lengths of time at either 4 or 37 °C from 0–60 min, followed by the addition of labeled ERE and a further incubation at 37 °C for 15 min. Preincubation at 4 °C had little effect on DNA binding in the absence or presence of ligands (data not shown), whereas preincubation at 37 °C gave differential DNA binding (Fig. 4A and D). In the presence of E2, HEG0 almost fully maintained its ability to bind to the ERE, and in the presence of OHT, a loss of only 20% in DNA binding was evident after 60 min. In the absence of ligand or the presence of ICI, however, HEG0 displayed a rapid reduction in the amount of complex formed even after only 5 min of preincubation at 37 °C (Fig. 4D). Similarly, preincubation of hERΔβ0 at 37 °C in the presence of E2 resulted in little reduction in DNA binding and a reduction of about 30% in DNA binding in the presence of OHT, while rapid loss was apparent in the absence of ligand or in the presence of ICI (Fig. 4A).

Next, we examined the effect of temperature on specific complex formation by performing the preincubations in the presence or absence of ligand at 4 °C for 30 min, followed by incubation in the presence of the ERE for varying lengths of time at 37 °C. A similar increase in DNA binding was observed...
for HEG0 and hERβ0 in the presence of E2 or OHT (Fig. 4B and E), DNA binding reaching maximum after incubation for 30 min. Incubation of HEG0 in the absence of ligand or the presence of ICI was maximal after 5 min. Interestingly in the presence of ICI, DNA binding by hERβ0 was similar to that observed in the presence of E2 and OHT. In the absence of ligand, DNA binding by hERβ0 or HEG0 was significantly lower than that observed in the presence of ligand.

As reported previously (25), when the preincubation and incubation steps were performed at 4 °C, DNA binding by HEG0 was indistinguishable in the absence of ligand or the presence of E2, OHT, and ICI although maximal binding was only reached after incubation for 4 h (Fig. 4F). When a similar experiment was performed using hERβ0, the results were essentially similar (Fig. 4C).

**hERβ Dimersize with hERα**—hERα and β are highly homologous, with 96% amino acid sequence identity in the DNA binding domain. We therefore wanted to determine whether whole cell extracts from COS-1 cells transfected with HEG0 and hERβ0 contained heterodimers in addition to homodimers. Heterodimer formation was studied using gel shifts since the HEG0-ERE and hERβ0-ERE complexes migrate at different positions in the gel (see Fig. 2) the presence of a complex at a position intermediate between the respective homodimeric complexes would be indicative of the presence of heterodimers.

 Indeed, transfected of HEG0 or hERβ0 alone resulted in single bands, whereas an additional complex was observed at an intermediate position when HEG0 and hERβ0 were co-expressed in COS-1 cells (Fig. 5A, compare lanes 2–4).

Heterodimer formation was further investigated using deletion mutants of HEG0, lacking the A/B region (HE19) or the ligand binding domain (HE15). Co-expression of hERβ0 and HE19 complex migrating at a position intermediate between the complexes observed for hERβ0 or HE19 alone (Fig. 5A, lane 4–6). Co-expression of hERβ0 and HE15 also resulted in an intermediate complex (compare lanes 4, 7, and 8), which was more clearly evident on longer runs (data not shown). Co-expression of HE81 (encoding amino acids 176–282 of hERα) also showed the presence of heterodimers, indicating that the DNA binding domain is sufficient for heterodimerization of hERα and β (Fig. 5A, lanes 9–11).

Ligand requirement for heterodimer formation was investigated using whole cell extracts of cells transfected with HEG0 and hERβ0 transfected either separately or together. Ligands were added 2 h prior to harvesting. Gel shift assays were performed as described for Fig. 2. Heterodimers were observed when HEG0 and hERβ0 were co-transfected, irrespective of the presence or absence of ligands, and the relative proportions of the respective homo- and heterodimers was similar for each sample (Fig. 5B).

**DISCUSSION**

The mechanisms of ERα function have been extensively studied in the decade following its cloning, and a great deal of attention has also been focused on the mechanisms of action of estrogen antagonists, particularly tamoxifen and ICI 164,384.
These studies have revealed that ERα activates gene expression by binding to EREs in responsive genes through the synergistic action of transcription activation functions AF-1 and AF-2. The partial agonist tamoxifen (or OHT) inhibits AF-2 but not AF-1. The mechanism of action of ICI 182,780, in vitro DNA binding by hERβ, is less adversely affected by temperature and, furthermore, indicate that ICI can “protect” hERβ from heat inactivation to some extent.

In summary, hERα and β bind specifically to the same DNA elements, and in vitro DNA binding characteristics are essentially similar for both in the presence of E2 or the partial antagonist 4-hydroxy-tamoxifen. In the absence of ligand or in the presence of the antagonist ICI 182,780, in vitro DNA binding by hERα is adversely affected at high temperature (37 °C). The mechanisms of this loss in DNA binding are unclear but are not due to degradation and may result from loss of suitable confirmation for DNA binding. While a similar effect was observed for hERβ when preincubation and incubation are performed at 37 °C, preincubation at 4 °C was sufficient for efficient in vitro DNA binding at 37 °C, suggesting that hERβ displays greater “conformational stability” in the absence of estrogens.

Amino acid sequence comparisons also indicated that ERα and β may heterodimerize. Our results using gel shift assays and immunoprecipitations show that hERα and β form heterodimers and that amino acids 176–282 of hERα are sufficient for heterodimerization. A strong dimerization function is present in the ligand binding domain of hERα, and indeed, heterodimer formation between full-length hERβ and hERα or HE19 (ΔA/B) was clearly more efficient than heterodimerization observed with HE81. As has previously been described for dimer formation by hERα (25), heterodimer formation between hERα and β was ligand-independent, similar ratios of homo- to heterodimer being observed in the absence of ligand or in the presence of E2, OHT, or ICI. Heterodimer formation between hERα and β means that in vivo they may cooperate in regulating estrogen-responsive gene expression in cell types in which they are co-expressed.

The importance of ERβ in vivo is as yet unclear. ERα knock-out mice display gross reproductive organ abnormalities and are infertile, indicating that ERα is sufficient for mediating the
effects of estrogens. It is however, possible that ERα and β act cooperatively, and knocking out ERα is thereby sufficient to produce the abnormalities observed. Alternatively (or in addition), it is possible that ERβ is more important than ERα in mediating prenatal estrogenic effects. Since ERα and β can bind to similar DNA elements together with cooperativity by heterodimer formation, the existence of a second ER gene opens up the possibility of differences in trans-activation of estrogen-responsive genes due to the presence of ERα and/or β. Differential responses to ligands as indicated by our study could also provide differential transcriptional activation by ERα and β.

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