Effect of Antagonists on DNA Binding Properties of the Human Estrogen Receptor in Vitro and in Vivo

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Functional analyses, performed with the estrogen receptor (ER) isolated from different sources or produced with various expression systems, led to contradictory results concerning the role of estrogen (E2) and antiestrogens in ER DNA binding. Here we report the DNA-binding properties of the human ER and show that the wild type ER (HEG0) binds in vitro to an estrogen response element (ERE) as a dimer, irrespective of the presence or absence of estrogen. We also show that the two antihormones, 4-hydroxytamoxifen (OHT, a partial ER agonist) and ICI 164,384 (a pure antagonist) do not impair HEG0 dimerization and DNA binding in vitro. Exposure of HEG0 to elevated temperature (37°C) in vitro results in a much faster reduction of its binding capacity to an ERE in the absence of ligand or in the presence of ICI 164,384 than in the presence of either E2 or OHT. The Gly to Val mutation at amino acid 400 present in the human ER that we initially cloned (HEO), is responsible for an even faster heat inactivation of unliganded receptor compared with HEG0 and largely accounts for the previously observed in vitro ligand-dependent DNA binding of ER. We also show that, as previously observed for OHT, ICI 164,384 does not prevent ER binding to an ERE in vivo, even though ICI 164,384 acts as a pure antagonist for transcriptional activation by ER. We discuss these results in the context of a ligand-dependent interaction between the C-terminal region E, which contains the ligand-binding domain, and the N-terminal A/B region, which contains the activation function AF-1. (Molecular Endocrinology 9: 579-591, 1995)

INTRODUCTION

Steroid hormones regulate initiation of transcription of target genes by binding to nuclear receptors that interact with specific DNA sequences called response elements. Cloning of the cDNAs encoding receptors for estrogens, progestins, androgens, glucocorticoids, mineralocorticoids, and ecdysteroids has revealed that these receptors belong to a gene superfamily that includes vitamin D3, retinoic acid, and thyroid hormone receptors, peroxisome proliferator-activated receptors, developmental regulatory proteins in Drosophila, and several vertebrate orphan receptors whose possible ligands are as yet unknown (1-7).

Nuclear receptors can be divided into six regions (A to F) according to the degree of amino acid sequence conservation (2, 8). The 66-68 amino acid-long core of region C is highly conserved throughout the family and is responsible for response element-specific DNA binding (Refs. 9-21 and references therein). The length and sequence of the N-terminal A/B region of the various nuclear receptors is variable and, in a number of cases, a transcriptional activation function (AF-1, previously called TAF-1) has been mapped to this region (10, 22-32). Region E, which is also well conserved, contains the ligand-binding domain and a hormone-inducible transcriptional activation function (AF-2, previously called TAF-2) (Refs. 10, 23-25, 27-29, and 32-35 and references therein). In the case of the estrogen receptor (ER) it has been shown that region E is also involved in nuclear localization (Refs. 36 and 37 and references therein), association with the heat shock protein hsp90 in vitro (38), and dimerization of the receptor (39, 40). A weaker dimerization domain is also associated with region C of the ER (17, 18, 39). Region D, which is much less well conserved, also contributes to formation of the ER-hsp90 complex in vitro (see Ref. 38 for references) and to nuclear localization (37).
The mechanism by which ligands control the function of nuclear receptors remains to be clarified. The classical model for steroid hormone action proposes that binding of the ligand by the receptor induces structural changes leading to nuclear localization, DNA binding, and activation or repression of transcription. In the case of ER, which is nuclear even in the absence of estrogen (E2) (37), E2 is thought to induce dissociation of the complex composed of ER and hsp90, allowing ER to bind DNA. Salt treatment has also been reported to induce binding of unliganded ER to DNA (41). In vitro functional analyses performed with the human ER (hER) initially cloned by us (HEO cDNA) (42, 43) and expressed in either HeLa cells (39, 44), Xenopus oocytes (45) or yeast (46), or produced by in vitro transcription/translation (47), have shown that binding of ER to an estrogen response element (ERE) was hormone-dependent, and also that E2 induced the formation of receptor dimers (39). However, DNA binding of HEGO produced in SF9 cells (baculovirus system) was reported to be hormone-dependent in the presence, but not in the absence, of Mg++ and that this dependency was more pronounced at higher temperatures (48). We have previously reported that HEO contains an artifactual point mutation resulting in the substitution of a glycine at position 400 by a valine (Gly400 → Val; see Ref. 49) and shown that this point mutation decreased the hormone binding at 25°C, but not at 4°C. On the other hand, it has been reported that the wild type hER, HEGO (also designated hER400) produced either by in vitro transcription/translation or in transfected COS-1 cells, binds strongly to an ERE even in the absence of hormone (42, 50). Hormone-independent formation of ER-ERE complexes has also been observed with extracts (51) and purified receptor (52, 53) from calf uterus, rat uterine extracts (54, 55), mouse uterine extracts (56, 57), and with mouse ER produced either by in vitro transcription/translation (26, 58), in transfected COS-1 cells (58, 59), or in SF9 cells infected with a recombinant baculovirus (40, 58). In contrast, ligand-inducible DNA binding, using HEGO produced by in vitro transcription/translation or in SF9 cells, has also been reported (60). In vivo hormone-independent association of hER with MCF-7 cell chromatin has been observed (61), thus suggesting that E2 may not be required for ER binding to the ERE, but only to convert the receptor bound to DNA into a transcriptionally active form. Ligand-dependent and ligand-independent ERE binding has also been reported for hER and Xenopus ER, respectively, by using a promoter interference assay (62, 63). However, in yeast, chromatin structure studies of an estrogen-responsive promoter have indicated that hER DNA binding is ligand-dependent in vivo (64), and it was shown that estrogen may be required to promote DNA binding at low, but not at high, receptor concentration (65). Furthermore, in vivo genomic footprinting experiments have indicated that occupation of the ERE present in the apoVLDLII promoter region is hormone-dependent (66), thus suggesting that the hormone is required for the ER to bind to the ERE.

The nonsteroidal antiestrogen OHT has been shown to affect hER activity by interfering with the transcriptional activation function AF-2 located in the hormone-binding domain, rather than by inhibiting DNA binding (23, 28), but contradictory results have been obtained with the antiestrogen ICI 164,384 (called hereafter ICI). In contrast to OHT, which does not inhibit AF-1 and acts as a mixed agonist/antagonist, ICI behaves as a pure antagonist in vivo (28), and it has been proposed that it inhibits DNA binding by affecting receptor dimerization (40). However, in vitro DNA binding studies using HEO-containing HeLa extracts (44) or purified calf uterine ER (52, 53) treated with ICI in vitro did not reveal such an inhibition. Inhibition of binding of HEGO to an ERE has been observed when the receptor was exposed to ICI in vivo, but not in vitro (60). Arbuckle et al. (58) have shown that ICI could prevent receptor dimer formation but could not disrupt dimeric complexes. It has also been reported that ICI reduces the cellular ER content in the mouse uterus and in the estrogen-responsive mouse cell line TM4 (67). Furthermore, Dauvois et al. (59) have shown that ICI reduces cellular mouse ER content by increasing its turnover. However, in vivo cross-linking studies in MCF-7 cells (61), transfection experiments using a modified ER (68), or promoter interference assay (62) have indicated that ER may bind to DNA in the presence of ICI. The above discrepancies in the effect of E2 and ICI on the ability of the ER to bind to an ERE may reflect species differences in LhTs or merely differences in the methods used to overproduce and extract ER and in testing DNA binding. We therefore decided to systematcally study the in vitro ERE binding characteristics of wild type hF (HFG0) and of the mutant form (HEO), produced and extracted in various ways. We show here that the binding capacity of wild type hER to an ERE in vitro is more sensitive to elevated temperature in the absence of ligand than in the presence of either E2 or OHT. Moreover, the pure antagonist ICI 164,384 does not protect the receptor from rapidly losing its ability to bind DNA at elevated temperatures. Interestingly, the HEO mutation (HEO) appears to be much more temperature-sensitive than HEGO, which accounts for the differences in DNA binding properties between the wild type and this mutant hER. We also report that transformation of the receptor by high salt (HS), E2 and/or elevated temperature is not required for efficient binding of wild type hER to an ERE in vitro. All of these results prompted us to investigate the dimerization properties of the wild type FR (HFG0); we show that it binds to an ERE in vitro as a dimer, irrespective of the absence or presence of either E2, OHT, or ICI. Finally, we present experiments suggesting that the presence of the antiestrogen ICI 164,384 does not inhibit binding of the ER to an ERE in vivo, but rather results in ERE-receptor-complexes in which both AF 1 and AF 2 are unable to activate transcription.
RESULTS

Binding of hERval400 (HE0), but not of hERrel400 (HEGO), to an ERE in Vitro Is Ligand-Dependent at 37 C

Due to the previously reported differences in E2 binding by the wild type hER (HEGO) and the mutant form (HE0) (49), we compared DNA binding in vitro, in the presence or absence of different ligands. HE0 and HEGO were produced either in a rabbit reticulocyte lysate (RRL) system, or in transformed yeast Saccharomyces cerevisiae cells. HE0, produced by in vitro transcription/translation, or extracted in an HS buffer (containing 0.4 M KCl) from transfected COS-1 or yeast cells, was first preincubated in gel shift buffer (containing KCl to a final concentration of 80 mM) at 4 C for 30 min in the presence or absence of either E2, OHT, or ICI. Radiolabeled ERE was then added to the reaction for 20 min at 37 C, before the receptor-ERE complexes were analyzed by gel electrophoresis mobility shift assay (EMSA) (Fig. 1). In agreement with our previous results and those of others (see Introduction), HE0 bound DNA in the presence, but not in the absence, of E2. A retarded complex was also seen in the presence of OHT, but it migrated more slowly than the E2-ER-ERE complex. DNA binding by HE0 was also observed in the presence of ICI, but at much lower level than with either E2 or OHT. The ICI-ER-ERE complex migrated at a position intermediate between those obtained in the absence of ligand and in the presence of OHT. The complex obtained in the presence of ICI migrated at a position intermediate between those observed in the presence of E2 and OHT. The complex obtained in the presence of ICI migrated at a position intermediate between those obtained in the absence of ligand and in the presence of OHT, showing that ICI was indeed bound to ER (Fig. 1B, compare lane 4 with lanes 1 and 3, lane 8 with lanes 5 and 7, and lane 12 with lanes 9 and 11). This latter difference in mobility was more clearly seen using the mutant HEG19 in which the A/B region of HEG0 is deleted (see Fig. 1C and D).

Effect of Temperature on in Vitro ERE Binding by hER

The effect of temperature on ER DNA binding was investigated using in vitro untreated or ligand-treated HS extracts from COS-1 cell transfected with HEG0. After a preincubation at 4 C or at 37 C for 15 min, radiolabeled ERE was added for 15 min at 37 C, and the complexes formed were analyzed by EMSA (Fig. 2A). In the presence of E2 or OHT, preincubation at 4 C or 37 C had little effect on DNA binding (Fig. 2A, compare lanes 3 and 4, and lanes 5 and 6), whereas in the absence of ligand, or in the presence of ICI, incubation at 37 C resulted in greatly reduced DNA binding (compare lanes 1 and 2 and lanes 7 and 8). Western blot analysis showed that the reduced DNA binding in the latter cases was not due to degradation of HEG0 during incubation (Fig. 2B). Similar results were obtained with HEG0 produced in rabbit reticulocyte lysate (data not shown). When HE0 was used in this assay the results were similar to those shown in Fig. 1, whether the preincubation was carried out at 4 C or at 37 C (data not shown).

The above results suggest that hER is inactivated in some way by preincubation at 37 C in the absence of ligand and in the presence of the antagonist ICI. To confirm this, untreated or ligand-treated HEG0-containing COS-1 cell HS extracts were preincubated for varying lengths of time at either 4 C or 37 C, at which point labeled ERE was added, and the reactions were further incubated at 37 C for 20 min. Preincubation at 4 C had no effect on DNA binding by HEG0 (data not shown).
Fig. 2. Temperature-Dependent Loss of DNA-Binding Capacity of HEGO in the Absence of E$_2$ or OHT and in the Presence of ICI

A, EMSA performed with HEGO expressed in COS-1 cells. HS-WCE were preincubated (30 min) in the absence of hormone (lanes 1–2), in the presence of 100 nM E$_2$ (lanes 3–4), 100 nM OHT (lanes 5–6), or 100 nM ICI (lanes 7–8) at either 4 C or 37 C (as indicated). The incubation step with the radiolabeled ERE was at 37 C for 30 min. B, Western blot analysis of the extracts treated as indicated above using anti-ER F3 monoclonal antibody (Materials and Methods).

shown). At 37 C HEGO retained its capacity to bind to an ERE even after a long preincubation at 37 C in the presence of either E$_2$ or OHT, whereas in the absence of hormone or in the presence of ICI only about 30% of the complexes were formed after 15 min of incubation at 37 C (Fig. 3A). This loss of DNA binding ability by HEGO in the absence of E$_2$ was irreversible, since addition of E$_2$ after the preincubation step at 37 C did not increase DNA binding (data not shown).

To examine the effect of temperature on specific complex formation, the preincubation step was performed at 4 C, and the DNA-binding step was performed at either 37 C or at 4 C. At 37 C, maximum binding was observed after a 30-min incubation with the ERE in the presence of E$_2$ or OHT. However, binding was maximal after 5 min in the absence of hormone or in the presence of ICI and, at best, represented less than 40% of the maximal binding observed in the presence of E$_2$ (Fig. 3B). By contrast, when the binding reactions were performed at 4 C, complex formation was similar in the absence or presence of ligand (Fig. 3C). The presence of 5–10 mM Mg$^{2+}$ during the reactions did not modify the hormone independence of the binding of HEGO to the ERE (data not shown). Although the time taken for maximal binding to be achieved was much greater, more complex was formed at 4 C than at 37 C using the same amount of HEGO-containing extracts (compare panels B and C of Fig. 3). The reduction of ERE-binding capacity by heat treatment in the presence of hormone was even more pronounced for HE0 than for HEGO. Figure 4A represents the amount of ERE-hER complexes formed with untreated or hormone-treated HE0 and HEGO-containing COS-1 cell extracts after preincubation for 15 min to 1 h at 37 C, followed by a 2-h DNA-binding step at 4 C before EMSA analysis. Preincubation at 37 C had no effect on DNA binding by E$_2$-treated HE0 and HEGO. However, in the absence of ligand, a 15-min preincubation at 37 C resulted in a 85% reduction of the DNA-binding capacity of HE0, and only in a 60% reduction for HEGO; after a 30-min preincubation at 37 C, complex formation with HE0 was almost totally abolished, whereas it was reduced by 75% in the case of HEGO. In contrast, by performing the preincubation and incubation reactions at 4 C, DNA binding was hormone-independent for HE0, as well as for HEGO (Fig. 4B and data not shown).

Effect of Ionic Strength on Binding of ERE by hER In Vitro

The binding of hER to DNA may be affected by pretreatment with salt (see Introduction). To investigate whether an HS treatment of the receptor is required for DNA binding, HEGO-transfected COS-1 cells were extracted in a low salt (LS) buffer (50 mM KCl). Salt was added to aliquots of these extracts to a final concentration of 400 mM KCl for 30 min at 4 C. Salt-treated and untreated extracts were then preincubated in the absence or presence of ligands in EMSA buffer (80 mM KCl final concentration) at 4 C for 30 min before incubation with labeled ERE (2 h at 4 C for Fig. 5A or various times for Fig. 5B). HEGO extracted in LS buffer, bound DNA in the absence of hormone, irrespective of an additional HS treatment (Fig. 5A, compare lanes 1 and 5). Furthermore, the presence of ligands (E$_2$, OHT, ICI) did not significantly affect the amount of complex formed (compare lanes 2–4 to lane 1, and lanes 6–8 to lane 5, in Fig. 5A) or the kinetics of its formation (see Fig. 5B).

In fact, HEGO, whether extracted in HS or LS buffer, bound DNA in the absence of hormone, provided that the final salt concentration in the binding reaction was at least 50 mM. Optimum DNA binding occurs at 100 mM KCl, even though the receptor was present as a 9S form at this salt concentration (data not shown). These results indicate that salt transformation of the receptor is not required for ERE binding.
Antiestrogens and ER DNA Binding Activity

**Fig. 3. Effect of Temperature on in Vitro Complex Formation**

EMSA was performed with HEG0 extracted in HS DNA-binding buffer from transfected COS-1 cells. Hormones were added in vitro as described in Fig. 2. The preincubation step was performed for various times at 37°C (A) as indicated or at 4°C for 30 min (B and C). The incubation step was performed for 20 min at 37°C (A) or for various times (as indicated) at 37°C (B) or 4°C (C). After electrophoresis, the specific retarded complex and the unbound ERE were excised and the radioactivity counted. The maximum amount of complex formed in presence of E₂ in panel B is taken as 100% for all three sets of experiments.

**ER Binds in vitro to DNA as a Dimer in the Absence and Presence of E₂, OHT, or ICI**

We analyzed the dimeric status of hER bound in vitro to an ERE using extracts of transfected COS-1 cells expressing HEG0 and/or HEG19, a mutant of HEG0 from which the N-terminal region A/B has been deleted (see Fig. 1D and Ref. 26). Hormones or antihormones were added to the cell culture medium for 1 h as indicated (Fig. 6A). The HEG19 complex (Fig. 6A, lanes 9–12) migrated faster than that formed with HEG0 (Fig. 6A, lanes 1–4), and the amount of complex formed correlated with the amount of receptor present in the extracts as determined by Western blot analysis (data not shown). When HEG0 and HEG19 were coexpressed in COS-1 cells, an additional complex with an intermediate migration was observed, corresponding to the binding of HEG0/HEG19 heterodimers. The presence of ligands did not significantly affect the amount of heterodimeric complexes formed (Fig. 6A, lanes 5–8, and data not shown). These results demonstrate that the ER binds DNA as a dimer, irrespective of the absence or presence of the various ligands tested. We also tested whether the addition of the ligands in vitro had an influence on the formation of heterodimeric complexes when cell extracts containing unliganded HEG0 or HEG19 were mixed in vitro. In all four cases, similar amount of heterodimeric complexes were formed (Fig. 6A, lanes 5–8, and data not shown). That ICI did not prevent the formation of dimeric complexes in vitro was further supported by the result obtained at a different HEG0/HEG19 ratio (Fig. 6B, lane 5–10).

Similar results were obtained with HEG0 produced in Sf9 insect cells using a baculovirus expression system. HEG0 bound to DNA both in the presence or in the absence of E₂ or ICI. By mixing HEG0 produced in Sf9 cells with HEG19 produced in COS-1 cells, two strong complexes corresponding to homodimers were seen, whereas only a faint heterodimeric complex could be detected (lanes 4 and 7, Fig. 6C). Addition of E₂ or ICI to the extracts, before or after mixing HEG0 with HEG19, did not affect the formation of het-
The ER Binds to DNA in the Presence of ICI in Vivo

The above results show that hER can bind an ERE in the presence of ICI in vitro. We therefore investigated whether hER could also bind DNA in the presence of ICI in vivo. We first constructed a chimeric receptor containing an ICI-insensitive heterologous transcriptional activation function designated VP16-HEG19, in which the acidic transcriptional activating domain of VP16 is associated with the ER DNA-binding and ligand-binding domains. The activity of this chimeric receptor was tested in HeLa cells using the ERE-G-CAT reporter gene containing a 17 mer response element (17M) for the yeast transactivator GAL4, followed by a response element for the ER (ERE), which was itself located upstream of the β-globin promoter region (17M/ERE-G-LUC, see Fig. 8A). This reporter plasmid was activated by HEGO in the presence of E2, and some transcriptional activity was also observed in the absence of E2 addition (Fig. 8B). As previously reported, this activity was suppressed upon addition of ICI or OHT (Fig. 8B and data not shown; see Refs. 28 and 49). No induction of transcriptional activity by HEGO was observed using the 17M/GRE-G-CAT reporter plasmid in which the ERE has been replaced by a glucocorticoid response element to which ER cannot bind (Fig. 8, A and B, and data not shown). Both reporters were constitutively activated by the chimeric activator GAL4-VP16 bound to the 17M site (Fig. 8C; see also Ref. 25). By coexpressing GAL4-VP16 and HEGO, we expected full activation of transcription by GAL4-VP16, if the receptor could not bind DNA in the presence of ICI. On the other hand, if HEGO could bind to the DNA in the presence of ICI, but could not transactivate, we expected that transactivation by GAL4-VP16 might be inhibited because binding of HEGO could interfere with GAL-VP16 DNA binding or interaction with the transcription machinery due to the proximity of the binding sites. To exclude the possibility that such a transcriptional inhibition could result from squelching and/or heterodimer formation, and not from ER binding to the ERE in presence of ICI, we used the reporter plasmid 17M/GRE-G-CAT to which ER cannot bind. HeLa cells cotransfected with 17M/ERE-G-LUC and 17M/GRE-G-CAT, together with HEGO and/or GAL4-VP16, were grown in the absence of hormone (charcoal-stripped medium) or in the presence of E2 or ICI for 24 h. Luciferase and chloramphenicol acetyltransferase (CAT) activities in various extracts were determined (Fig. 8C). Neither E2 nor ICI significantly affected luciferase or CAT expression in GAL-VP16-transfected cells. On the other hand, when HEGO was cotransfected, the expression of the 17M/ERE-G-LUC reporter was 3-fold decreased in the presence of ICI, whereas it was 5-fold increased in the

As expected, OHT, which does not prevent the binding of the ER to DNA in vivo but cannot activate the AF-2 activation function (Ref. 28 and references therein), acted as an agonist on VP16-HEG19, while blocking the activity of HEG19. In the presence of ICI, HEG19 was inactive, as expected (28), but VP16-HEG19 was a strong activator (Fig. 7B), thus indicating that it was bound to DNA.

Although the above results suggest that the ER can bind to an ERE in the presence of ICI in vivo, it could not be excluded that in the wild type receptor, the presence of the A/B region may result in a different structure for which ICI could prevent DNA binding. To demonstrate that HEGO can indeed bind DNA in the presence of ICI, we constructed a chimeric luciferase reporter gene containing a 17 mer response element (17M) for the yeast transactivator GAL4, followed by a response element for the ER (ERE), which was itself located upstream of the β-globin promoter region (17M/ERE-G-LUC, see Fig. 8A). This reporter plasmid was activated by HEGO in the presence of E2, and some transcriptional activity was also observed in the absence of E2 addition (Fig. 8B). As previously reported, this activity was suppressed upon addition of ICI or OHT (Fig. 8B and data not shown; see Refs. 28 and 49). No induction of transcriptional activity by HEGO was observed using the 17M/GRE-G-CAT reporter plasmid in which the ERE has been replaced by a glucocorticoid response element to which ER cannot bind (Fig. 8, A and B, and data not shown). Both reporters were constitutively activated by the chimeric activator GAL4-VP16 bound to the 17M site (Fig. 8C; see also Ref. 25). By coexpressing GAL4-VP16 and HEGO, we expected full activation of transcription by GAL4-VP16, if the receptor could not bind DNA in the presence of ICI. On the other hand, if HEGO could bind to the DNA in the presence of ICI, but could not transactivate, we expected that transactivation by GAL4-VP16 might be inhibited because binding of HEGO could interfere with GAL-VP16 DNA binding or interaction with the transcription machinery due to the proximity of the binding sites. To exclude the possibility that such a transcriptional inhibition could result from squelching and/or heterodimer formation, and not from ER binding to the ERE in presence of ICI, we used the reporter plasmid 17M/GRE-G-CAT to which ER cannot bind. HeLa cells cotransfected with 17M/ERE-G-LUC and 17M/GRE-G-CAT, together with HEGO and/or GAL4-VP16, were grown in the absence of hormone (charcoal-stripped medium) or in the presence of E2 or ICI for 24 h. Luciferase and chloramphenicol acetyltransferase (CAT) activities in various extracts were determined (Fig. 8C). Neither E2 nor ICI significantly affected luciferase or CAT expression in GAL-VP16-transfected cells. On the other hand, when HEGO was cotransfected, the expression of the 17M/ERE-G-LUC reporter was 3-fold decreased in the presence of ICI, whereas it was 5-fold increased in the

The ER Binds to DNA in the Presence of ICI in Vivo

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erodimers (compare lanes 5 and 6 with lane 4, and lanes 8 and 9 with lane 7).

Taken all together, these results indicate that the ER binds to DNA in vitro as a dimer irrespective of the absence or presence of E2, OHT, or ICI. Note also that less heterodimeric complexes were formed when HEG0 and HEG19 were mixed in vitro when compared with complexes formed with extracts containing coexpressed HEG0 and HEG19 (compare Fig. 6B, lanes 1–4 to Fig. 6A, lanes 5–8; see also Fig. 6C), indicating that some dimers preexisted in the extracts before binding to the ERE.

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presence of E$_2$. Under the same conditions, the expression of the 17M/GRE-G-CAT reporter was unchanged, thus indicating that the inhibition of 17M/ERE-G-LUC expression observed in the presence of ICI was due to the specific DNA binding of HEGO-ICI complexes. Note that in the absence of ligand, the presence of HEGO did not result in any inhibition of transcriptional activation by GAL-VP16. This may indicate that the ER cannot bind DNA in the absence of ligand in vivo. Alternatively, this lack of inhibition could result from ligand-independent transcriptional activity of HEGO (see above, Fig. 8B), which may be due to the presence of residual estradiol in the charcoal-stripped cell culture medium.

**DISCUSSION**

**The Wild Type ER (HEGO) Binds an ERE in Vitro as a Dimer Irrespective of the Presence or Absence of E$_2$, OHT, or ICI 164,384**

We have studied here the DNA binding and dimerization characteristics of the hER in the absence or presence of estradiol and of two antiestrogens, OHT and ICI. We show that the cloned, wild type receptor hER$_{gy400}$ (HEGO), produced in several systems (in vitro transcription/translation, transiently transfected COS-1 cells, recombinant baculovirus-infected Sf9 cells, or transformed yeast cells) binds to the X. vitellogenin A2 gene palindromic ERE in vitro in the absence of any ligand. These results are in agreement with those reported previously using the rat, mouse, calf, and human ER (26, 40, 47, 50-59). Furthermore, in the absence of any ligand, more heterodimeric complexes are formed with the ERE when the full-length ER, HEGO, and the A/B-truncated mutant, HEG19, are coexpressed in COS-1 cells, than upon mixing in vitro extracts containing HEGO expressed either in COS-1 cells or in Sf9 cells, with extracts containing HEG19 expressed in COS-1 cells. These observations, which suggest that ER dimers preexist in cell extracts in the absence of hormones, are in keeping with the previous study of Skafar (70) who reported that ER dimers exist in solution in the absence of any ligand. It has been proposed that the association of hER with hsp90 (migrating as a 9S complex on a sucrose gradient) prevents its binding to DNA in the absence of hormone, which would occur only after transformation into a 4-5 S form generated in vitro by treatment with HS (0.4 M KCl), or elevated temperature (25 C) and estradiol (41). We found that none of these conditions were required for HEGO to bind DNA in vitro, since constitutive binding was observed with or without salt pretreatment.
also noteworthy that even at 4 C, the kinetics of DNA binding is the same in the presence or absence of E2, OHT, or ICI as indicated. CAT activity was assayed after normalization for β-galactosidase activity from the reference plasmid pCH110 (Materials and Methods).

Fig. 7. ICI Is an Agonist in HeLa Cells for a Chimeric ER Containing an Acidic Activating Domain

A, Schematic representation of the receptors and reporter gene. The numbers refer to amino acid positions for transactivators and to nucleotides for the reporter. B, CAT assay experiment using extracts from HeLa cells transfected with ERE-TATA-CAT (1 pg) together with either the parental vector pSG5 (50 ng, lanes 1-4), HEG19 (50 ng, lanes 5-8), or VP16-HEG19 (50 ng, lanes 9-12). Cell cultures were maintained in the absence (−) or presence (+) of E2 (10 nM), OHT (100 nM), or ICI (100 nM) as indicated. CAT activity was assayed after normalization for β-galactosidase activity from the reference plasmid pCH110 (Materials and Methods).
reflect a stabilization of the dimerization interface upon ligand binding.

The hER Binds DNA in Vivo in the Presence of E₂, OHT, and ICI

We have previously shown that while E₂ can induce the activity of the two activation functions (AF-1 and AF-2) of hER, OHT selectively blocks the activity of AF-2, thus accounting for its cell- and promoter-specific mixed agonistic/antagonistic effect (Refs. 25 and 28 and references therein). In principle, the full antagonistic action of ICI in vivo (Ref. 28 and references therein) could be due either to an inhibition of binding of the ER to DNA or to an inhibitory effect on both AF-1 and AF-2.

Two different approaches were used here to demonstrate that ICI does not inhibit the binding of HEG0 to DNA in vivo. We first showed that a chimeric receptor (VP16-HEGO), containing the VP16 acidic activating domain instead of the N-terminal activation function AF-1, stimulates transcription in the presence of ICI. Therefore, ICI does not inhibit DNA binding of the VP16 chimeric receptor, in agreement with results obtained with other chimeric constructs (68). Note that these results also indicate that ICI does not prevent dimerization of HEG19 in the HEG19-VP16 chimera.

However, results obtained with chimeric receptors do not prove that ICI also allows dimerization and DNA binding of the wild type receptor, as deletion and insertion of amino acid sequences may alter the properties of the receptors. To investigate whether ICI-liganded HEG0 could bind to DNA, we used an assay in which its DNA binding would repress activation of transcription of a reporter gene by the GAL-VP16 activator. Constitutive transcriptional activation by GAL-VP16 bound to a GAL4 17M response element was shown to be decreased by ICI-liganded HEG0 through binding to an ERE located downstream of the 17M. Since we excluded that this decrease could be due to heterodimer formation or to transcriptional interference/squelching (71, 72), our results clearly show that ICI-liganded hER can selectively bind to an ERE. This
AF-1 is prevented in the case of ICI, but not of OHT, to bind DNA (64). Thus, whether the unliganded ER and the N-terminal-activating domain. The function of specific manner, which is dependent on both the ligand to act as a pure transcriptional antagonist (Refs. 28 to an ERE in vitro in the absence of any agonistic or active effect of estrogen against heat denaturation of ER. to an ERE in the presence of OHT, which prevents tant HE0 (see Ref. 49) was clearly reflecting a protec- 

Conclusion
Our present results demonstrate that the ER can bind to an ERE in vitro in the absence of any agonistic or antagonistic ligand. The previously reported requirement of estrogen (39) with the initially cloned ER mutant HE0 (see Ref. 49) was clearly reflecting a protective effect of estrogen against heat denaturation of ER. In this respect, OHT appears to be as efficient as estradiol, whereas the pure antagonist ICI is much less effective, which may, at least in part, account for the decrease in ER levels observed in ICI-treated cells (59, 69). We have previously reported that the ER can bind to an ERE in the presence of OHT, which prevents activation of transcription by the activation function AF-2, while AF-1 can still transactivate in a promoter- and cell context-dependent manner (28, 73), thus accounting for the mixed agonistic/antagonistic effect of OHT. Interestingly, we show here that ICI does not prevent ER binding to an ERE, even though it is known to act as a pure transcriptional antagonist (Refs. 28 and 74, and references therein). It is noteworthy that the chimeric receptor VP16-HEG19, in which the ER N-terminal A/B region has been replaced by the acidic activator VP16, does transactivate in the presence of both OHT and ICI. Therefore, binding of different ligands to the ER C-terminal ligand-binding domain affects the function of the N-terminal region in a specific manner, which is dependent on both the ligand and the N-terminal-activating domain. The function of AF-1 is prevented in the case of ICI, but not of OHT, while both OHT and ICI allow transactivation by the VP16 activating domain of VP16-HEG19. Clearly there is some cross-talk between the otherwise seemingly independent N- and C-terminal functional domains of the ER. Our study also shows conclusively that an antagonist that would prevent binding of the ER to DNA remains to be found. Whether such an antagonist would be more or less efficient than ICI in blocking the expression of estrogen target genes (e.g., those expressed in breast cancer) is an interesting open question.

MATERIALS AND METHODS

Recombinants
HE0 (75), HEG0 (49), HEG19 (28), GAL-VP16 (25), pYERE1/HE0 (48), and pYERE1/HEG0 (49) have been described. VP16-HEG19 was constructed as follows: the EcoRI-KpnI fragment from VP16(N) (27) containing the nuclear localization signal from SV40 T antigen and amino acids 411–490 of VP16, and the KpnI–EcoRI fragment from HE80 containing amino acids 177–595 of hER (gift of S. Mader) were cloned into the EcoRI site of pSCS5. The Eagl-BamHI region containing the valine at position 400 was replaced by the corresponding fragment isolated from pSG5-HEG0. The reporter plasmid ERE-TATA-CAT is as described (25). 17M/ERE-G-CAT has been constructed by cloning an oligonucleotide containing the GAL4 17 mer binding site and glucocorticoid response element sequences into the XbaI site of pGCGAT (23). 17M/ERE-G-LUC (gift of M. Saunders) was constructed by cloning the HindIII-BglII fragment isolated from 17M/ERE- G-CAT (25) containing the promoter region, into the unique HindIII site of pSV40ALA5’ (76), using a synthetic BglII–HindIII oligonucleotide.

In Vitro Expression of hER
cDNA sequences of HE0 and HEG0 were subcloned into the EcoRI site of Bluescribe M13+ vector downstream of the T7 promoter (9, 49). Recombinants were linearized with BamHI. Capped mRNA was synthesized using 5 μg linearized DNA template by T7 polymerase (50 U) at 37 C for 1 h in a buffer containing 10 mM Tris HCl, pH 7.8, 8 mM MgCl2, 50 mM NaCl, 1 mM spermidine, 10 mM dithiothreitol (DTT), 0.5 mM ATP, 10 mM dGTP and 0.2 mM cap structure analog [m7G(5)ppp(5)G], and 100 U RNasin in a final volume of 100 μL. The RNA synthesized was quantitated by trichloroacetic acid precipitation of an aliquot (4.5 μL) of a transcription mixture that included [32P]CTP. Five hundred nanograms of capped mRNA were translated in the presence of nonlabeled amino acids using rabbit reticulocyte lysate (Promega, Madison, WI) in a final volume of 50 μL. Incubations were carried out at 30 C for 60 min. The amount of hER synthesized was quantitated by Western blot analysis and by hormone binding assay as described previously (46).

Cell Transfection
COS-1 and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium without phenol red, supplemented with 5% dextran-coated, charcoal-treated fetal calf serum (77). Cells were transfected using the calcium phosphate coprecipitation technique at 50% confluence in 9-cm petri dishes with a total of 15 μg DNA [Bluescribe M13+ (Stratagene, La Jolla, CA) was used as carrier DNA (25, 28)]. The amount of expression vector transfected for gel retardation experiments was 5 μg. Hormones (E2, 10 nM; OHT, 100 nM; ICI, 100 nM) were added 1 h before harvesting. For CAT and luciferase assays the amount of expression vector and reporter gene is indicated in the figure legends. The amount of reference plasmid pCH110 (Pharmacia, Piscataway, NJ) used was 2 μg.
Gel retardation/shift assay (EMSA)

Gel retardation assays (20 μl) contained 1–10 μg extract, 2.5 μg poly(dI-dC), and 50,000 cpm of 5'-[32P]-end-labeled double-stranded ERE (5'-TCGAGCAAAGTCAGGTCACAGT-GACCTGATCAAT-3') in 10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM DTT, 80 mM KCl, 5% glycerol (vol/vol), 0.5 mM phenylmethylsulfonylfluoride, and 1.25 mg/ml of leupeptin, pepstatin, chymostatin, antipain, and aprotinine. Mixtures were preincubated for 30 min on ice in the presence or absence of 100 nM estrogen and to the unbound ERE were excised, and the radioactivity was determined by liquid scintillation counting.

Western Blot Analysis

Western blot analysis was performed as described (46) using the monoclonal antibody F3 (69) directed against the F region of the hER.

β-Galactosidase, CAT, and Luciferase Assay

β-Galactosidase activity was determined by the method of Herbomel et al. (78). CAT assays containing extracts corresponding to 5 U β-galactosidase for ERE-TATA-CAT reporter plasmid and to 5 μg WCE for 17M/CRE-G-CAT reporter plasmid were performed as described (28). Luciferase activity was determined on 20 μg WCE with a LKB luminometer (LKB Instruments, Rockville, MD) (76).

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