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Structural differences between the hormone and antihormone estrogen receptor complexes bound to the hormone response element

(DNA–receptor interaction/gel retardation)

MICHÈLE SABBAH, FABRICE GOUILLEUX, BRIGITTE SOLA, GÉRARD REDEUILH, AND ETIENNE-EMILE BAULIEU*

Unité de Recherches sur les Communications Hormonales, Institut National de la Santé et de la Recherche Médicale, U.33, and Faculté de Médecine, Laboratoire Hormones, Université Paris-Sud, 94275 Bicêtre Cedex, France

Contributed by Etienne-Emile Baulieu, October 5, 1990

ABSTRACT **To investigate the molecular mechanism(s) by which the estrogen receptor (ER) modulates transcription, we compared the structures of receptor complexes containing estradiol, the agonist diethylstilbestrol, and the antagonist ICI164,384. The binding of ICI164,384 to nontransformed 8–9S ER does not preclude the salt-induced dissociation of the 90-kDa heat shock protein and releases the transformed homodimeric 5S ER as classically observed in the presence of agonist. We report that calf ER binds to the estrogen response element of the *Xenopus* vitellogenin A2 gene in either the absence or presence of hormone, agonist, or antagonist. These binding interactions were highly sequence- and receptor-specific. These findings indicate that ligand binding *in vitro* is not absolutely required for dimerization or specific DNA binding of the ER. As demonstrated by gel retardation assays, the ICI164,384–ER complex bound to the response element displays a slower mobility than complexes formed in the presence of estradiol or agonist. This difference in electrophoretic mobility is suggestive of a conformational change in the complex induced by the ligand. An exchange experiment demonstrated that this alteration of the structure is reversible. We suggest that ICI164,384 induces conformational modifications within the ligand-binding domain of the receptor that do not prevent binding to the response element but could fail to promote subsequent events required for gene transcription.**

Estrogen regulation of gene transcription in target cells is mediated by specific high-affinity intracellular receptor proteins. Upon activation by hormone binding, the receptor interacts specifically with a cis-acting DNA sequence called the estrogen response element (ERE), which is usually located upstream from the gene promoter and displays enhancer properties (1–4). The first step in steroid hormone action resides in its binding to receptor. In turn, it has been proposed that hormone binding activates receptor function, leading to formation of functional hormone–receptor complexes.

The molecular mechanism(s) by which antiestrogen exerts its antagonist activity is still unknown. The recent availability of the first pure antiestrogen compound, ICI164,384, now allows a detailed biochemical analysis of antiestrogen activity. The steroid antagonist ICI164,384 is entirely devoid of uterotrophic activity in rats and mice and completely blocks the stimulatory effect of estradiol (5). At the molecular level, ICI164,384 binds with high affinity to estrogen receptor (ER) and acts as a pure antagonist of ER-induced transcription of a vitellogenin ERE–thymidine kinase promoter–chloramphenicol acetyltransferase gene construct (6). Thus, this behavior suggests that ICI164,384 is unable to promote one or more critical steps leading the receptor to act as a transcription-activating factor.

We report here that the binding of ICI164,384 to nontransformed 8–9S ER does not preclude the salt-induced dissociation of the 90-kDa heat shock protein (hsp90) and releases the transformed homodimeric 5S ER. The receptor—in the absence of ligand or complexed with estradiol (E_2), agonist (diethylstilbestrol, DES), or antagonist (ICI164,384)—binds tightly to the ERE of the *Xenopus* vitellogenin A2 gene. However, by gel retardation (band shift assay), the ICI164,384–ER (ICI–ER) complex, bound to ERE, exhibits a slower migration than the complex formed in the presence of E_2 or agonist. Intermediate shifted complexes are observed in the absence of ligand. The modification in electrophoretic mobility is attributed to a ligand-induced conformational change of complexes that differs when an agonist or an antagonist is bound. A model is proposed in which the antagonist ICI164,384 might act at a stage subsequent to ER binding to ERE.

MATERIALS AND METHODS

[6,7-^{3}H]E_2 (specific activity, 78 Ci/mmol; 1 Ci = 37 GBq) was from CEA (Gif sur Yvette, France); nonradioactive E_2 was a gift from Roussel–Uclaf (Romainville, France). DES was from Sigma. Acrylamide, *N*,*N*′-methylenebisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, and ammonium persulfate were from Bio-Rad. Klenow DNA polymerase was obtained from Boehringer Mannheim. [α-^{32}P]dCTP (3000 Ci/mmol) was from Amersham and poly(dI-dC) from Pharmacia.

Oligodeoxynucleotides. Synthetic oligonucleotides corresponding to the wild-type ERE (EREwt, 5′-GATCCAAAGTCAGGTCACAGTGACCTGATCAAAGTTA-3′; palindrome is underlined) and the mutated ERE (EREm,5′-GATCCAAAGTCAGaTCACAGTGAtCTGATCAAAGTTA-3′) were purified by gel electrophoresis (20% polyacrylamide, 8 M urea). The probes were prepared by annealing complementary strands in 50 mM Tris, pH 7.5/1 mM spermidine/10 mM $MgCl_2$/1 mM dithiothreitol by heating to 85°C and cooling to room temperature over a period of 3 hr and then labeled with [α-^{32}P]dCTP by using the Klenow fragment of DNA polymerase.

Band Shift Assay. Binding reaction mixtures (14 μl) containing 1 mM sodium phosphate (pH 7.2), 0.1 mM EDTA, 2 μg of poly(dI-dC), 2 μg of sonicated sperm salmon DNA, and

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Abbreviations: ER, estrogen receptor; ERE, estrogen response element; EREwt, wild-type ERE; EREm, mutant ERE; E_2, estradiol; DES, diethylstilbestrol; hsp90, 90-kDa heat shock protein; ICI–ER, ICI164,384–ER.

*To whom reprint requests should be addressed.

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0.05 ng (1–2 × 10^4 cpm) of probes were incubated with variable amounts of ER-containing extract at room temperature for 30 min. A pre-electrophoresis (20 mA for 30 min) was performed and protein–DNA complexes were separated from protein-free DNA by nondenaturing electrophoresis (7) in 6% polyacrylamide gels (29:1 acrylamide/*N,N'*-methylenebisacrylamide weight ratio). Gels were run at room temperature in 0.25× TBE (50 mM Tris/50 mM boric acid/1 mM EDTA) at 20 mA. Gels were dried and autoradiographed.

Preparation of ^{3}H-Labeled Ligand–ER Complexes. Calf uteri were homogenized in 3 volumes of 10 mM Tris·HCl/1 mM dithiothreitol/20 mM sodium molybdate, pH 7.5 at 25°C. The cytosol was obtained as described (8). The receptor was purified by DEAE-Sephacel chromatography (8) before labeling with tritiated ligand.

Glycerol Gradient Ultracentrifugation. Glycerol gradients were prepared and ultracentrifugation was performed as described (8).

RESULTS

Sedimentation Analysis of ER Labeled with [^{3}H]ICI164,384. It has been proposed that binding to hormone, but not to antihormone, facilitates the dissociation of hsp90 from the 8–9S nontransformed glucocorticosteroid (9) and progesterone (10) receptors. *In vitro*, the 8–9S ER complexed with E_2 can be transformed even in the presence of molybdate, at 4°C, by increasing the ionic strength (11, 12). Under low-salt conditions, the ER labeled with [^{3}H]ICI164,384 sedimented as an 8–9S peak at 4°C in glycerol gradients containing molybdate. When the [^{3}H]ICI–ER, treated or not by 0.4 M KCl for 1 hr at 4°C, was centrifuged through a high-salt (0.4 M KCl) glycerol gradient containing molybdate, it sedimented at 5S (Fig. 1). Thus, even at reduced temperature, ICI164,384 did not stabilize or retard salt-induced dissociation of the nontransformed 8–9S ER.

Effects of Ligand on Receptor Binding to ERE. We investigated the influence of agonists and antagonists on the binding of ER to its target DNA *in vitro*. A 37-base-pair synthetic oligonucleotide containing the palindromic sequence of the *Xenopus* vitellogenin A2 gene hormone response element (EREwt, ref. 13) was used as probe in gel shift assays.

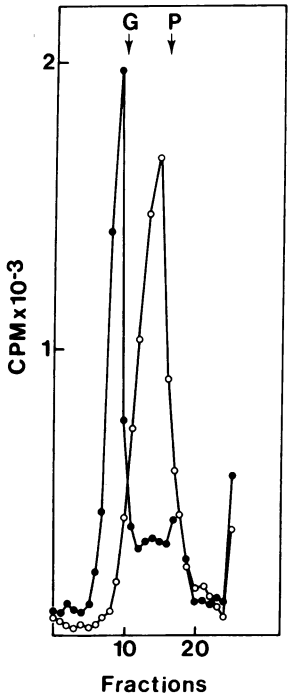


FIG. 1. Glycerol gradient centrifugation. Samples containing 8–9S [^{3}H]ICI–ER complexes were analyzed by sedimentation in a 10–35% glycerol gradient with low salt (●) or high salt (0.4 M KCl) (○). The samples were centrifuged in a Beckman SW60 rotor at 220,000 × *g* for 15 hr. Arrows indicate the sedimentation of internal standards: G, glucose oxidase (7.9 S); P, peroxidase (3.6 S).

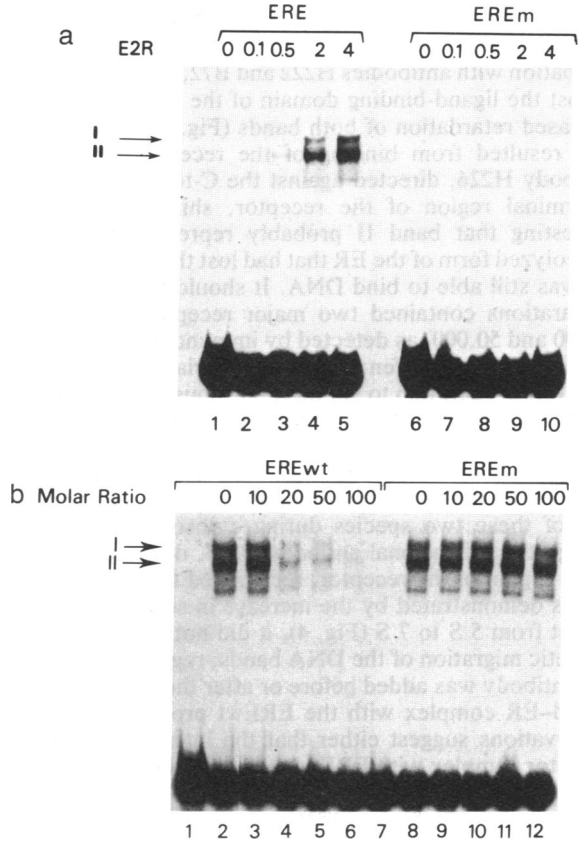


FIG. 2. Sequence-specific binding of ER to the ERE. (*a*) Binding reaction mixtures contained 0.05 ng of EREwt (lanes 1–5) or EREm (lanes 6–10) probe and 0.1, 0.5, 2, or 4 ng of receptor bound to E_2 (E2R). (*b*) Unlabeled competitor oligonucleotides were included in the binding reaction mixture prior to addition of E_2–ER (4 ng). The molar ratio of competitor over the labeled probe (EREwt, 0.05 ng) is indicated above each lane. Protein–DNA complexes were separated from protein-free DNA by polyacrylamide gel electrophoresis and visualized by autoradiography.

Fig. 2*a* presents data showing the interaction of ER (complexed with E_2) and the ERE target. The 3'-end-labeled oligonucleotide was incubated with increasing amounts of ER-containing extract (lanes 1–5). Two retarded bands (I and II) were seen for the highest amounts of extract (lanes 4 and 5). To demonstrate the specificity of the interaction, two different experiments were performed. First, the same amounts of extract were incubated with an oligonucleotide (EREm) identical to EREwt except for two point mutations reported to lead to a loss of estrogen responsiveness in transient-transfection assays (14). No retarded bands were seen, suggesting that the retarded bands I and II resulted from binding of ER to the EREwt probe (Fig. 2*a*, lanes 6–10). Second, EREwt and EREm were used to compete for the ER binding to the labeled EREwt probe. A 20-fold excess of EREwt efficiently displaced retarded bands I and II (Fig. 2*b*, lanes 1–6), whereas even a 100-fold excess of EREm did not compete with the EREwt probe (lanes 7–12). These experiments demonstrated a strong specificity of the protein for the DNA target but did not prove that the protein that bound to the oligonucleotide was the ER.

To demonstrate that the retarded bands were due to ER–ERE interaction, the 3'-end-labeled oligonucleotide was incubated with extract that had been passed through a ligand-specific affinity column under conditions where >98% of the receptor was retained on the column (15). As expected, no retarded bands were seen after nondenaturing gel electrophoresis (data not shown). In addition, we incubated

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anti-ER monoclonal antibodies (16) with the receptor before or after incubation with the labeled oligonucleotide probe. Incubation with antibodies H222 and B72, which are directed against the ligand-binding domain of the receptor, led to an increased retardation of both bands (Fig. 3), indicating that both resulted from binding of the receptor to the ERE. Antibody H226, directed against the C-terminal part of the N-terminal region of the receptor, shifted only band I, suggesting that band II probably represented a partially proteolyzed form of the ER that had lost the epitope for H226 but was still able to bind DNA. It should be noted that our preparations contained two major receptor species of M_r 66,000 and 50,000, as detected by immunoblotting or affinity labeling with tamoxifen aziridine, in variable concentrations from one preparation to another. Previous work showed that the M_r 65,000 form of the ER was recognized by monoclonal antibodies H222, H226, D547, and B72, while the M_r 50,000 species did not react with H226 (15, 17). The inclusion of protease inhibitors had a weak influence on the representation of these two species during cytosol preparation. Although the monoclonal antibody D547, directed against the hinge region of the receptor, recognized the transformed 5S ER as demonstrated by the increase in sedimentation coefficient from 5 S to 7 S (Fig. 4), it did not affect the electrophoretic migration of the DNA bands, regardless of whether the antibody was added before or after the interaction of the ligand–ER complex with the EREwt probe (Fig. 3). These observations suggest either that the interaction of the E_2–receptor complex with EREwt may alter the conformation of the ER, causing the release of the antibody, or that EREwt blocks access of the antibody to the hinge portion of the receptor and may compete with the monoclonal antibody for binding.

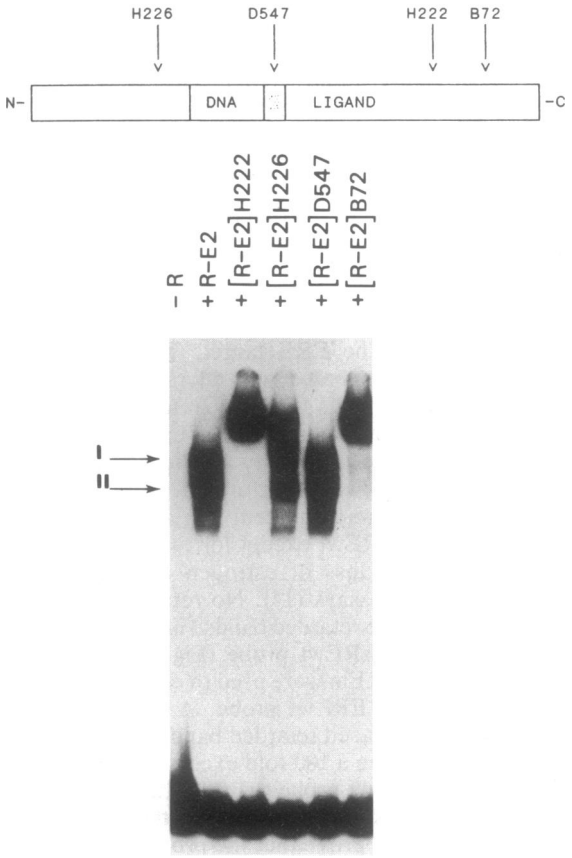


FIG. 3. Identification of ER–ERE complexes by specific antibodies. Binding reaction mixtures contained 0.05 ng of probe EREwt and 4 ng of receptor complexed to [^{3}H]E_2 (R–E2). Monoclonal antibodies (1 μg) were included in the binding reaction mixture and further incubated for 2 hr at 4°C. Protein–DNA complexes were separated as in Fig. 2. Above the autoradiogram is a schematic of the wild-type ER showing the map of recognition sites for four monoclonal antibodies used in these assays.

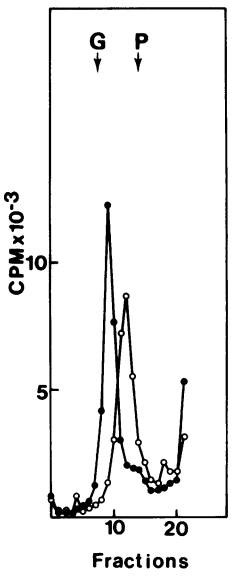


FIG. 4. Density gradient sedimentation analysis of ER with the monoclonal antibody D547. A fraction containing 8–9S [^{3}H]E_2–ER complexes was adjusted to 0.4 M KCl and then transformed by heating at 28°C for 45 min (○). The 5S ER was incubated for 2 hr at 4°C with a 300-fold excess of monoclonal antibody D547 (●). Aliquots were centrifuged at 200,000 × *g* for 17 hr at 4°C in a high-salt 10–35% glycerol gradient in a Beckman SW60 rotor. Arrows indicate the sedimentation of standard proteins: G, glucose oxidase (7.9 S); P, peroxidase (3.6 S).

Taken together, these results clearly show that the bands observed in the gel shift assay reflect a very specific binding of the ER to the ERE. To examine the effect of the ligand on receptor–DNA binding, we used the agonist DES and the antagonist ICI164,384. Although a similar doublet of DNA complexes was detected (Fig. 5) independently of the presence of the ligand and its agonist or antagonist effects, several reproducible and consistent differences were observed. The ER–EREwt complex formed in the presence of ICI164,384 displayed a lower mobility (by ≈10%) than the ER–EREwt complex formed in the presence of E_2 or DES. However, ICI164,384 often gave complexes of lower intensity, which can be attributed to the instability of the ICI–ER complexes observed in solution (unpublished data). Interestingly, intermediate shifted complexes were observed in the absence of ligand (Fig. 5). The presence of liganded receptors in preparations used for DNA-binding assays could not be detected as assayed by exchange techniques. Thus, *in vitro*, in the presence or in the absence of any ligand, the ER binds specifically to the ERE. The antagonists tamoxifen, hydroxytamoxifen, tamoxifen aziridine, and RU39411 also gave complexes of slower electrophoretic mobilities than E_2 and DES (data not shown), as previously observed for hydroxytamoxifen (18, 19) and LY117018 (20).

The modification in electrophoretic mobilities of the ER–ERE complexes suggests a ligand-induced conformational change that differs depending on whether an agonist or an antagonist is bound. To test whether the effect of the ligand was reversible, we performed experiments in which E_2 and ICI164,384 bound to receptor were exchanged for ICI164,384 and E_2, respectively. In these experiments, electrophoresis was performed under conditions allowing the free oligonucleotide to run off the gel, in order to increase the electrophoretic migration of retarded bands. The two retarded bands due to the interaction of [^{3}H]E_2–labeled ER with the ERE (Fig. 6, lanes 1–3) were converted by exchange of E_2 for ICI164,384 to slower migrating bands of the same electrophoretic mobilities as those detected when the ERE was incubated with [^{3}H]ICI164,384-labeled ER (compare lane 4 with lanes 5–7). On the other hand, the retarded bands visualized with [^{3}H]ICI164,384 were converted, after exchange of ICI164,384 for E_2, into complexes of the same electrophoretic mobilities as seen with [^{3}H]estradiol (compare lanes 4, 5, 6, and 7 with lanes 8, 1, 2, and 3). Since the

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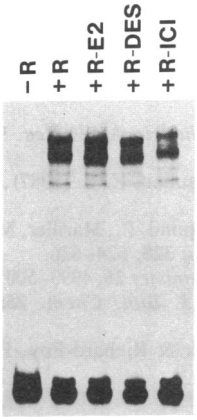


FIG. 5. Effect of ligand on binding of calf ER to its EREwt. Binding reactions were carried out after preincubation of the ER with no added receptor (−R), no added hormone (+R), 10 nM E_2 (+R-E2), 10 nM DES (+R-DES), or 10 nM ICI164,384 (+R-ICI). Binding reaction mixtures contained 0.05 ng of EREwt probe and 4 ng of receptor. Protein–DNA complexes were separated as in Fig. 2.

migration of band II, which lacks the N-terminal part of the receptor, was also slower in the presence of antagonist, we conclude that the antagonist ICI164,384 induces a reversible conformational change, probably in the ligand-binding domain of the receptor, different from that induced by an agonist. Nevertheless, we did not detect any difference in the interactions of anti-ER monoclonal antibodies with the receptor labeled with E_2 (Fig. 3) or ICI164,384 (data not shown) prior to or after incubation with the labeled oligonucleotide probe.

Moreover, hormone-free ER complexed to the ERE remained able to bind agonist or antagonist and a similar

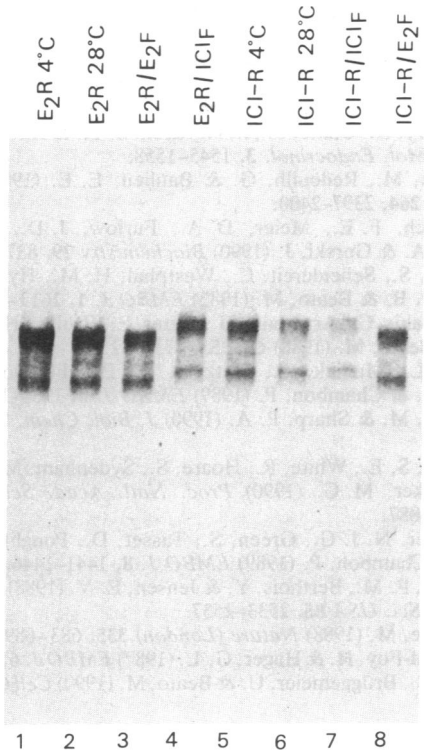


FIG. 6. Dependence of the nature of ligands on the electrophoretic mobilities of ER–EREwt complexes. Samples containing ER were incubated with [^{3}H]E_2 or [^{3}H]ICI164,384 (10 nM) for 17 hr at 4°C in order to label the ER binding sites. An aliquot of each fraction was equilibrated at 28°C (3 min) and dissociation of [^{3}H]E_2 or [^{3}H]ICI164,384 was initiated by addition of free (F) nonradioactive ICI164,384 (lanes 4 and 7) or E_2 (lanes 3 and 8). The exchange reactions were carried out for 40 min. Stability controls were always performed simultaneously by keeping aliquots at 4°C and 28°C (lanes 1, 2, 5, and 6). Binding reaction mixtures contained 0.05 ng of EREwt probe and 4 ng of receptor. Electrophoresis was continued until protein-free DNA had run off the gel.

difference in electrophoretic mobility of the respective complexes was detected (data not shown).

DISCUSSION

Two nonexclusive hypotheses have been proposed to explain the mechanism of action of steroid antagonists: the steroid competes for the hormone-binding site but (*i*) fails to induce the transformation of the receptor and traps it in a non-DNA-binding form, probably through interaction with hsp90 (9, 21), or (*ii*) provokes the transformation of the receptor but fails to promote its transcriptional activity (6, 22–24). At the molecular level, ICI164,384 competes with E_2 for E_2 binding sites and, as reported here, ICI164,384 does not stabilize the nontransformed 8–9S receptor form. In intact cells, upon addition of ICI164,384, antagonist–receptor complexes become tightly bound to the nucleus (6). Thus, it is unlikely that the antiestrogen acts by stabilization of the 8–9S heterooligomer, thereby preventing the release of the hsp90. These data suggest that a step unrelated to DNA binding by the receptor accounts for the antiestrogen activity of ICI164,384.

We report here that, *in vitro*, calf uterus ER binds specifically to the ERE in the presence or absence of hormone, agonist, or antagonist. Two retarded bands can be visualized by gel shift assays, independently of the presence of ligand. We have shown that these two complexes are specific for the ERE and for the receptor. Monoclonal antibodies H222, H226, D547, and B72, which are directed against different epitopes along the receptor molecule (see ref. 16 and diagram in Fig. 3), show that the slower complex is formed by the intact receptor, whereas the faster complex represents a proteolyzed form of the receptor, always present in our preparations, that has lost the N-terminal part but remains able to form a dimer and bind DNA. These findings indicate that in the absence of ligand, the receptor adopts a dimeric structure and is able to fold into a conformation that recognizes the ERE, and therefore, *in vivo* binding to DNA cannot definitively be excluded. Previous findings have suggested that the formation of dimer occurs in the absence of ligand and DNA binding (25). Although these results are consistent with previous reports showing that the mouse (18) and the rat (26) ER, the glucocorticosteroid receptor (27), and the progesterone receptor (28) are able to bind to the hormone response element in the absence of hormone, they contrast with the hormone dependence of human ER dimerization and DNA binding (19). However, ligand-dependent induction of dimerization and DNA binding have been reported for an ER clone containing a point mutation in the ligand-binding domain, $Val^{400} \rightarrow Gly^{400}$, which decreases its stability and affinity for E_2 under conditions used for DNA-binding assays (29). In contrast, during the completion of this manuscript, work by Brown and Sharp (30) was published indicating that the same human [Gly^{400}]ER produced in a baculovirus expression system binds the ERE in the absence of hormone. Therefore, there is no obvious explanation for the observed differences in the ligand dependence of receptor–DNA binding.

In vivo, the presence of hormone is required for tight nuclear binding. The effect of hormone would be to induce the dissociation of hsp90 and to lead to an active hormone–receptor complex. In contrast, *in vitro*, the dissociation of hsp90 could occur during the incubation step with the ERE, even in the absence of hormone. We have been unable to determine a substantial difference in the kinetic parameters of ER binding to the ERE in the presence of agonist or antagonist. Our results suggest that differences in the binding affinities may be too small and cannot be determined by the method used in our experiments. It is likely that kinetic effects do not account for the antagonist activity of ICI164,384. Thus, mechanistically, our results suggest that

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the antihormone mimics the first steps of hormone action, leading to the release of the homodimeric structure of the receptor, which in turn is able to bind to the ERE, but in a different manner. These results differ from those of Fawell *et al.* (31), who suggested that ICI164,384 cannot induce dimerization and DNA binding of the mouse ER produced in the baculovirus expression system, in contrast to the *in vitro* translated receptor (18). The ER–EREwt complexes formed in the presence of ICI164,384 displayed a lower mobility than complexes formed in the presence of hormone or agonist (Fig. 6). This difference in electrophoretic mobility can be attributed to a conformational change of complexes induced by the ligand, as demonstrated by exchange. Moreover, since the electrophoretic migration of the faster retarded band, due to the proteolyzed form having lost the N-terminal part of the receptor, became slower in the presence of ICI164,384, it is likely that the conformational alteration affects the ligand-binding domain of the receptor.

These observations can be related to previous results showing that *in vivo*, the hormone, but not an antihormone, can induce a transcription activation function present in the ligand-binding domain (6). It has been suggested that the transcription activation function can be attributed to a three-dimensional folding of the ligand-binding domain (32) rather than to specific protein sequences, acidic "blobs," or amphipathic helix. The results presented here support this hypothesis and suggest that the three-dimensional folding of the hormone-binding domain induced by the ligand probably exhibits a difference in the ionic charge at the surface of the complexes. These findings are also in agreement with a report (33) showing that the binding of antiestrogens modifies the immunoreactivity of the ER toward monoclonal antibody H222, suggesting a receptor protein conformational change induced by the ligand.

The mechanisms by which the receptor modulates the rate of transcription *in vivo* are unknown. They probably involve receptor interaction with transcription factors and/or RNA polymerase itself (34) or alteration of the chromatin structure (35, 36). We propose that the antagonist ICI164,384 induces conformational modifications of the receptor that do not preclude binding to the ERE but fail to promote events needed for gene transcription.

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