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Inhibition of estrogen receptor–DNA binding by the "pure" antiestrogen ICI 164,384 appears to be mediated by impaired receptor dimerization

(transfection/gel shifts/antibodies/tamoxifen/baculovirus)

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ABSTRACT **Many estrogen-antagonist and -agonist ligands have been synthesized, some of which have proved clinically important in the treatment of hormone-dependent breast tumors and endocrine disorders. Here we show that the "pure" antiestrogen ICI 164,384 inhibits mouse estrogen receptor–DNA binding *in vitro*. The effects of this steroid on DNA binding can be overcome by addition of an anti-receptor antibody whose epitope lies N-terminal to the receptor DNA-binding domain. Since this antibody is also capable of restoring DNA-binding activity to receptor mutants that either lack the dimerization domain or bear deleterious mutations within it, we propose that ICI 164,384 reduces DNA binding by interfering with receptor dimerization. In contrast, when complexed with the antagonist/partial agonist tamoxifen, the estrogen receptor is capable of binding to DNA *in vitro*, but tamoxifen does not promote the agonist-induced conformational change obtained with estradiol. The implications of these data are discussed in relation to the *in vivo* properties of these drugs.**

The effects of steroid hormones are mediated primarily via specific receptor proteins that function as ligand-inducible transcription factors. Receptor proteins recognize and bind to specific target sequences in responsive genes and subsequently regulate their expression (1–3). The binding sites for the estrogen receptor, termed estrogen response elements (ERE), have been characterized and shown to be based on an inverted repeat of the sequence TGACC (4). Preliminary work suggested that estrogen receptors exist and bind to DNA in the form of dimers (5, 6), and this has now been demonstrated directly for the human protein (7). We recently identified sequences involved in this dimerization function in the mouse estrogen receptor (MOR) and found that residues important for estradiol binding colocalize with this dimerization domain (8).

The steroid dependence of some breast cancers has been known for almost 100 years and both endocrine therapy and surgery (ovariectomy/adrenalectomy) have been used for the control of this disease (9). Soon after the discovery of a specific cellular receptor for estrogens it was shown that there were variations in receptor levels in breast tumors (10, 11). Of those tumors possessing detectable estrogen receptor (cytosols containing >10 fmol of receptor per mg of protein), >60% have proved to be responsive to endocrine therapy, whereas <5% of receptor negative (<10 fmol/mg) tumors respond (12). It was then proposed that estrogens could regulate the growth of tumor cells and could act, presumably via the receptor protein, as mitogens in these cells. An extensive range of antiestrogens has therefore been developed for the control of hormone-dependent breast tumors (12). Potentially useful compounds are tested in a variety of animal models, including rat uterotrophic assays, and for their ability to transform the receptor protein, measured by changes in sedimentation coefficients or affinity for DNA-cellulose. The primary effects of all these compounds are thought to be mediated via interaction with the estrogen receptor protein, but little is known of their mechanism of action at a molecular level. A number of clinically important antiestrogens are nonsteroidal and include derivatives of triphenylethylenes. One of these, tamoxifen (ICI 46,474), successfully used for the treatment of receptor-positive breast cancer, has proved to be predominantly antiestrogenic. However, tamoxifen is not a pure antagonist but is capable of inducing receptor transformation and can behave as an agonist in certain test systems and animal models (13–15). There has therefore been a considerable effort to develop "pure" antiestrogens that may prove to be more effective in the control of hormone-dependent breast cancer. Recently, a number of steroidal 7α-alkylamide compounds have been described that seem to behave as pure antagonists in all assays tested to date (16, 17). In this paper we show that a member of this family, ICI 164,384 (Fig. 1), inhibits the DNA binding of the MOR in an *in vitro* assay and provide evidence to suggest that this involves the inhibition of receptor dimerization.

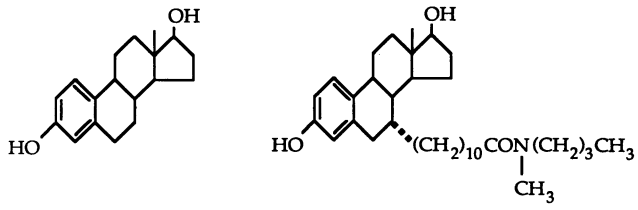

FIG. 1. Structures of 17β-estradiol (*Left*) and the estrogen antagonist ICI 164,384 (*Right*).

MATERIALS AND METHODS

Receptor Mutants. The isolation of a MOR cDNA clone, construction of a series of deletion and point mutants, and their transfer into pSP64 and pSP65 have been described (8, 18–20). Deletion mutants are described according to the receptor amino acids remaining (e.g., MOR121–384). Point mutants were constructed in the full-length protein (MOR1–599) and are named by amino acid and residue number

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Abbreviations: CAT, chloramphenicol acetyltransferase; ERE, estrogen response element; MOR, mouse estrogen receptor.

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mutated, followed by the substituting amino acid (e.g., L-511R represents mutation of leucine-511 to arginine).

Overexpression of the MOR in *Spodoptera frugiperda* Cells. Recombinant baculovirus containing a MOR cDNA under the control of the polyhedrin promoter was isolated following cotransfection of *S. frugiperda* cells with baculoviral DNA and the transfer vector p36C-MOR (ref. 21; S.E.F. and M.P., unpublished work). The cells were grown in suspension culture in TC100 medium (Flow Laboratories) containing 10% fetal bovine serum, plated out at 80% confluence, and infected with recombinant virus at a multiplicity of infection of 5–10 plaque-forming units per cell. After 72 hr the cells were harvested, collected by centrifugation, and frozen at −70°C. Whole cell extracts were prepared by resuspending the frozen pellets in high-salt buffer [0.4 M KCl/20 mM Hepes, pH 7.4/1 mM dithiothreitol/20% (vol/vol) glycerol containing bacitracin (1 mg/ml), aprotinin (5 μg/ml), pepstatin (5 μg/ml), and leupeptin (5 μg/ml)] passing the suspension five times through a 25-gauge needle, and removing debris by centrifugation at 50,000 × *g* for 20 min. Extracts were stored in aliquots at −70°C.

***In Vitro* Synthesis of Receptor.** Receptor mutant clones were linearized using *Sst* I, *Eco*RI, or *Hin*dIII as appropriate, and capped RNA was synthesized using SP6 polymerase (22) with the modifications described by Fawell *et al.* (8). This RNA was used at 15–30 ng/μl to prime synthesis of protein in a rabbit reticulocyte lysate (Promega) containing 0.1 mM $ZnCl_2$, a methionine-free amino acid mixture, and either 0.1 mM methionine or 1 μCi (37 kBq) of [^{35}S]methionine (Amersham; specific activity, 1000 Ci/mmol) per μl, as indicated. Translations were carried out at 30°C for 60 min.

Gel Shift Assay. DNA binding was assayed by electrophoretic mobility shift. Either 1–5 μl of *in vitro* translated receptor or 1 μl of whole cell extract from recombinant baculovirus-infected insect cells was preincubated for 15 min in 20 μl of binding buffer (10 mM Hepes, pH 7.4/50 mM KCl/1 mM 2-mercaptoethanol/20% glycerol) containing 1 μg of poly(dI-dC)·poly(dI-dC) and 100 μg of bovine serum albumin. Radiolabeled double-stranded oligodeoxynucleotide probe (1 ng) containing a consensus ERE sequence (20) was then added and the samples were incubated for a further 30 min at room temperature. Samples were analyzed by electrophoresis in 6.0% polyacrylamide (30% acrylamide/0.8% *N*,*N*′-methylenebisacrylamide stock solution) gels containing 0.5× TBE (45 mM Tris base/45 mM boric acid/1 mM EDTA). After electrophoresis in 0.5× TBE at 250 V for 60 min, gels were fixed for 15 min in 10% acetic acid/30% methanol, dried, and subjected to autoradiography.

Antibody Production. The peptide Cys-Gln-Gln-Val-Pro-Tyr-Tyr-Leu-Glu-Asn-Glu-Pro-Ser-Ala, corresponding to residues 130–142 of the MOR, was synthesized by standard solid-phase techniques, coupled to thyroglobulin via the added N-terminal cysteine, and used to immunize rabbits (8). The resultant antiserum (MP16) recognizes both mouse and human estrogen receptors on Western blots (S.E.F., unpublished work) and is able to immunoprecipitate the mouse protein translated *in vitro* (8). F(ab′)$_2$ fragments were prepared from MP16 IgG by digestion with pepsin [2.5% (wt/wt) overnight at 37°C] and removal of whole molecules by chromatography on protein A-Sepharose (Pharmacia). Monovalent F(ab′) fragments were then prepared by reduction with 2-mercaptoethanol (86 mM) and subsequent alkylation with iodoacetamide (2.5 mg/ml).

Transfection Studies. HeLa Ohio cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Cells were seeded for transfection at a density of 10^5 per 5-cm dish. After 24 hr the cells were fed with phenol red-free DMEM containing 5% charcoal-stripped fetal bovine serum and were transfected by calcium phosphate coprecipitation. A total of 10 μg of DNA was used per dish, including 1.0 μg of the MOR expression vector pJ3MOR, 5.0 μg of the reporter EREpBLCAT, and 1.0 μg of the control vector pJ3Luciferase (20). The luciferase activity arising from the control vector was used to normalize CAT activity for transfection efficiency. After 18 hr the cells were washed three times with phenol red-free DMEM and refed with phenol red-free DMEM containing 5% charcoal-stripped fetal bovine serum with steroids added as indicated. Cells were harvested 48 hr later and cell suspensions were assayed for chloramphenicol acetyltransferase (CAT) and luciferase activity (23, 24).

RESULTS

ICI 164,384 Functions as a Pure Antiestrogen. When tested in transient-transfection studies, the MOR is able to stimulate the expression of CAT activity from a reporter plasmid that includes an ERE. In addition to the stimulation of CAT activity in the presence of estrogens, there is appreciable receptor activity in the absence of any added steroid (Fig. 2). This probably represents the effect of residual steroid not removed by the charcoal stripping of the serum, but alternatively could indicate a hormone-independent activity of the receptor in this assay. We then examined the ability of the antiestrogens tamoxifen, 4-hydroxytamoxifen, and ICI 164,384 to inhibit receptor activity. Addition of increasing concentrations of tamoxifen or 4-hydroxytamoxifen to the medium resulted in a reduction in CAT activity, but even at 1 μM there was a significant, ≈5-fold, receptor-dependent induction (Fig. 2). Increasing concentrations of ICI 164,384, however, were able to abolish receptor activity, with 0.1–1 μM steroid reducing CAT activity to basal levels. Thus, in transfection experiments the steroidal antiestrogen ICI 164,384 appears to function as a pure antiestrogen, while the triphenylethylene derivatives tamoxifen and 4-hydroxytamoxifen do not completely abolish receptor activity and may possess partial agonist activity.

ICI 164,384 Inhibits Estrogen Receptor–DNA Binding *in Vitro*. Whole cell extracts containing the MOR were prepared from *S. frugiperda* cells infected with a MOR recombinant

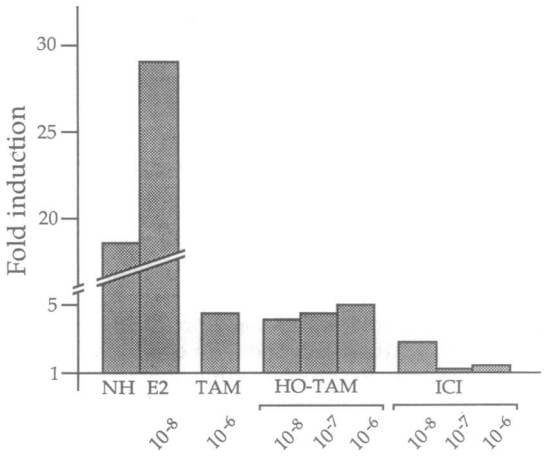

FIG. 2. ICI 164,384 behaves as a pure antiestrogen in transient-transfection experiments. HeLa Ohio cells were cotransfected with a MOR expression vector, an estrogen-responsive reporter plasmid encoding CAT, and a luciferase expression vector as an internal control. Cells were grown in phenol red-free medium containing 5% charcoal-stripped serum, supplemented with hormone as indicated, harvested after 48 hr, and assayed for CAT and luciferase activity. CAT values were normalized to luciferase activity and data are expressed as fold inductions over basal values in the absence of receptor. NH, no added hormone; E2, estradiol; TAM, tamoxifen; HO-TAM, 4-hydroxytamoxifen; ICI, ICI 164,384. Molar concentrations are indicated.

baculoviral vector and used in the gel shift assay. As previously shown for the *in vitro* translated receptor (8, 20), specific DNA binding was observed even in the absence of added steroid (Fig. 3). Upon addition of estradiol, DNA binding was not affected quantitatively but the protein–DNA complex migrated faster in the gel system. Similar results were obtained with the estrogen agonist diethylstilbestrol (20). In the presence of tamoxifen (or 4-hydroxytamoxifen) the receptor was also capable of binding specifically to the ERE probe, but in this case the mobility of the receptor–DNA complex was unaffected or slightly reduced. When the antagonist ICI 164,384 was added, a dramatic reduction was seen in receptor binding (Fig. 3). This is in contrast to results with *in vitro* translated receptor, where no inhibition was observed with this steroid (20). The reasons for this discrepancy are unclear; however, ligand binding assays failed to demonstrate any specific binding of ICI 164,384 to the *in vitro* synthesized protein, whereas specific binding could be seen with the baculoviral whole cell extracts (S.E.F., unpublished work).

Anti-Estrogen Receptor Antibody MP16 Restores Estrogen Receptor–DNA Binding in the Presence of ICI 164,384. Addition of a range of anti-estrogen receptor antibodies in the gel shift assay results in a "supershift," a complex with reduced mobility in the gel system, due presumably to the increased size of the complex with the antibody molecule. Surprisingly, however, in the presence of the antibody MP16 a specifically retarded complex was seen in the presence of the steroidal antagonist ICI 164,384 (Fig. 3). Indeed, receptor–DNA binding in the presence of ICI 164,384 and MP16 seemed as efficient and stable as in the presence of the other steroids. A range of other anti-receptor antibodies were then tested, but only MP16 demonstrated this property.

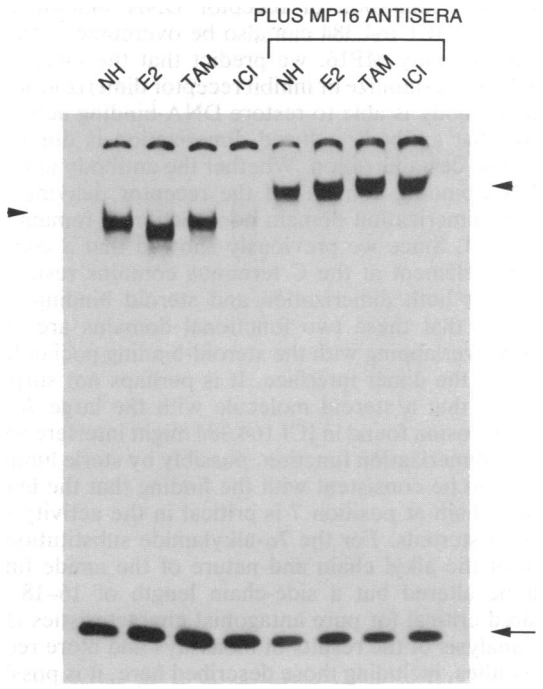

FIG. 3. Ligand effects on the ability of the MOR to bind DNA. *S. frugiperda* cells were infected with a MOR recombinant baculovirus and used to prepare whole cell extracts. These receptor extracts were preincubated with hormones and antiserum MP16 (1 μl) as indicated and then tested for DNA-binding activity in the gel shift assay. A 32-base-pair double-stranded oligonucleotide containing an ERE sequence was used as probe (20). The positions of the retarded complexes or free probe are marked with arrowheads and an arrow, respectively. NH, no added hormone; E2, 10 nM estradiol; TAM, 1 μM tamoxifen; ICI, 0.1 μM ICI 164,384.

Antibody MP16 Restores DNA Binding to Dimerization-Deficient Receptor Mutants. When the antiserum MP16 was added to a number of non-DNA-binding receptor mutants, DNA binding in the gel shift assay could be restored. In fact, all the mutants tested that both retained the epitope for this antiserum and had an intact DNA-binding domain were able to bind to an ERE with high affinity in the presence of MP16. This included receptor mutants that bore amino acid substitutions in the dimerization domain (Fig. 4, L-511R, lane 2) or large C-terminal deletions (Fig. 4, MOR121–384, lane 2) (8). The effect of this antibody was specific in that it was inhibited by addition of the peptide to which the antiserum was raised (Fig. 4, lanes 3), and the effect was not observed with preimmune serum or a variety of nonimmune sera. The monoclonal anti-estrogen receptor antibody H222 gave rise to a supershift of the wild-type receptor but did not restore DNA binding to mutant L-511R (Fig. 4, lanes 4). Western blotting confirmed that the epitope for this antibody was retained in mutant L-511R.

We suspect that the bivalent nature of the antibody allows antibody-induced receptor dimerization with the result that high-affinity DNA binding is restored. To test this hypothesis $F(ab')_2$ and F(ab') fragments were prepared from MP16 IgG. The Fc region of the IgG was removed by digestion with pepsin, resulting in a bivalent $F(ab')_2$ fragment, which on reduction yields monovalent F(ab') domains. When the wild-type receptor was incubated with the $F(ab')_2$ fragments a supershift was observed, but with the complex having increased mobility relative to the receptor–whole antibody complex, consistent with removal of the antibody Fc region. This antibody fragment retained the ability both to restore DNA binding to mutants L-511R and MOR121–384 and to

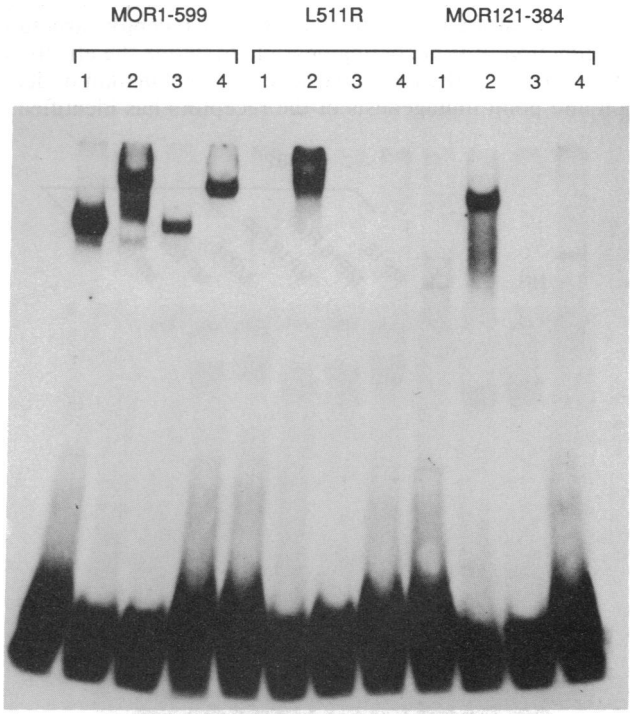

FIG. 4. Effect of anti-estrogen receptor antibodies on the DNA-binding activity of mutant receptors. Wild-type receptor (MOR1–599), the receptor point mutant L-511R, and the deletion mutant MOR121–384 were translated in rabbit reticulocyte lysates and tested for DNA-binding activity in the gel shift assay. The following were included in the preincubation before addition of the radiolabeled probe: no additions (lanes 1), 1 μl of anti-receptor antiserum MP16 (lanes 2), 1 μl of antiserum MP16 in the presence of 2 μg of a bovine serum albumin conjugate of the peptide (MOR130–142) used as the immunogen (lanes 3), 1 μl of monoclonal antibody H222 (lanes 4).

rescue the inhibition of wild-type binding in the presence of ICI 164,384 (Fig. 5). Addition of monovalent F(ab') fragments to the wild-type receptor resulted in a supershifted complex, indicating that these fragments were still able to recognize the receptor protein (Fig. 5). This complex did not migrate as a discrete band, however, but rather as a smear between the positions of the native receptor and receptor–$F(ab')_2$ complexes, presumably reflecting the instability of antibody–receptor interaction resulting from the loss of bivalency. However, the F(ab') fragments had lost the ability to restore DNA binding both to mutant L-511R or MOR121-384 (data not shown) and to the wild-type protein in the presence of ICI 164,384 (Fig. 5).

It was possible that the restoration of DNA binding by MP16 in the presence of ICI 164,384 was due to displacement of the steroid. This could have been brought about by an antibody-induced conformational change in receptor structure or by competitive binding of the antibody and steroid molecules. To test this theory the MOR was immunoprecipitated in the presence of labeled steroids. Whole cell extracts, containing ≈30 pmol of MOR per mg of protein, were prelabeled with [^{3}H]estradiol or [^{3}H]ICI 164,384, free steroid was removed with dextran-coated charcoal, and immunoprecipitations were carried out with preimmune serum, MP16, or the monoclonal antibody H222 (8). Both anti-receptor antibodies were able to specifically precipitate receptor-bound steroid for both ligands (data not shown); therefore, the binding of MP16 does not simply displace the bound ICI 164,384 steroid.

DISCUSSION

With the cloning of the genes for the steroid receptors and the wealth of information now available on receptor structure and function, it is becoming possible to define the effects of antiestrogens at the molecular level. A combination of deletion and point mutagenesis of the receptors has identified a central region (region C) (25–27) involved in DNA binding and C-terminal sequences (region E) encoding the steroid-binding and dimerization domains (7, 8, 25, 27). For the estrogen receptor transcriptional activation function has been mapped to two regions, a hormone-independent activity at the N terminus (regions A and B) and a hormone-dependent activity at the C terminus (20, 28). These regions have been shown to be active in the context of chimeric receptor proteins where receptor sequences were linked to heterologous DNA-binding domains of GAL4 or LexA (20, 29, 30).

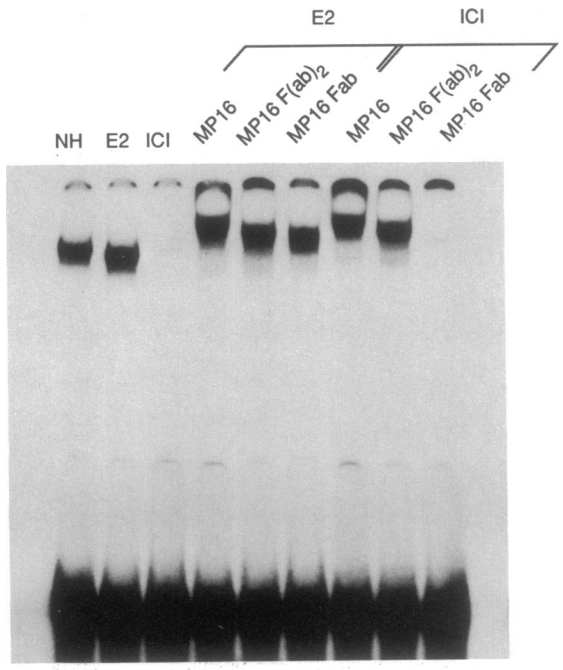

FIG. 5. Bivalent antibody MP16 is required to restore receptor–DNA binding in the presence of ICI 164,384. Whole cell extracts containing MOR were incubated with hormones and antibody fractions as indicated and tested for DNA binding in the gel shift assay. NH, no added hormone; E2, 10 nM estradiol; ICI, 0.1 μM ICI 164,384.

In this paper we have shown that the antiestrogen ICI 164,384, but not tamoxifen, inhibits the ability of the receptor to bind to DNA. The inhibitory effect of ICI 164,384 could be overcome by the antibody MP16, which is capable of restoring high-affinity DNA binding to mutant receptors that are defective for dimerization (8). Since only whole antibody molecules or $F(ab')_2$ fragments could restore DNA binding, we conclude that the epitope for this antibody is fortuitously positioned such that one bivalent antibody molecule induces or stabilizes the dimerization of two receptor monomers. This stoichiometry is supported by the equivalent mobility of the receptor–MP16 and receptor–monoclonal antibody H222 complex, since it has been shown that only one molecule of antibody H222 is able to bind to a receptor dimer (31). Antibody H222 does not, however, seem to possess the dimerization properties of MP16 (Fig. 4). The epitope for MP16 is N-terminal to the DNA-binding domain of the receptor and is presumed to be located close to the zinc-finger DNA-binding motifs in the folded protein. In the antibody-induced or -stabilized dimer, these zinc-finger motifs must be positioned similarly to the native receptor dimer in order to allow high-affinity, sequence-specific recognition and binding to an ERE.

Since the inhibition of receptor DNA binding by the antiestrogen ICI 164,384 can also be overcome in the presence of antibody MP16, we predict that the effect of this steroid is to destabilize or inhibit receptor dimerization. Also, as the antibody is able to restore DNA-binding activity, we assume that antibody-induced dimerization is dominant to the steroid destabilization. Whether the antibody aligns only the DNA-binding domains of the receptor, leaving the C-terminal dimerization domain nonfunctional, remains to be determined. Since we previously showed that a conserved sequence element at the C terminus contains residues important for both dimerization and steroid binding (8), we concluded that these two functional domains are at least partially overlapping with the steroid-binding pocket located at or near the dimer interface. It is perhaps not surprising, therefore, that a steroid molecule with the large 7α-alkylamide extension found in ICI 164,384 might interfere with the receptor dimerization function, possibly by steric hindrance. This would be consistent with the finding that the length of the side chain at position 7 is critical in the activity of this family of steroids. For the 7α-alkylamide substitutions, the length of the alkyl chain and nature of the amide function could be altered but a side-chain length of 16–18 atoms appeared critical for pure antagonist characteristics (17).

By analysis of the results of bioassays and more recent *in vitro* studies, including those described here, it is possible to define the effects of antiestrogens on the receptor in more precise terms. It has been shown that with a fusion protein consisting of the GAL4 DNA-binding domain linked to the hormone-binding domain of the human receptor, estrogens both promote DNA binding and activate the transactivation function contained within these sequences (29). In contrast, while tamoxifen also seems able to promote efficient DNA binding, it does not induce the transactivation domain (29). When estrogen agonists such as estradiol or diethylstilbestrol are added to the receptor in gel shift assays, a ligand-induced

conformational change is observed with an increase in the mobility of the receptor–DNA complex (ref. 20 and Fig. 3). In the presence of tamoxifen or 4-hydroxytamoxifen, however, the mobility of the receptor–DNA complex is somewhat decreased (Fig. 3). We have proposed that this difference reflects changes in receptor structure on binding of these ligands that correlate with transactivation-competent or -incompetent receptor states, respectively (20). Since tamoxifen will promote the binding of the receptor to an ERE *in vivo*, it has been proposed that activation of transcription could occur via the N-terminal domain (20, 32). The agonist/antagonist properties of this antiestrogen therefore may depend on the relative roles of the two receptor transactivation domains. In certain cell types or in the context of responsive promoters where the N-terminal domain can function independently, tamoxifen would be expected to behave as a weak agonist. In this respect the antiglucocorticoid/progestin RU486 may behave similarly to tamoxifen. RU486 has been reported to inhibit receptor transformation and behave as a pure antagonist by some groups (33). However, studies by Chambon and colleagues (28) using a GAL4–glucocorticoid receptor C-terminus chimera seem to indicate that RU486 will promote DNA binding although transactivation is not stimulated.

In contrast, in the presence of the antiestrogen ICI 164,384 we see little or no receptor DNA-binding activity, and we predict that this reflects reduced protein dimerization. Clearly in this situation neither receptor transactivation domain would be expected to be functional. This is in agreement with the lack of receptor transformation (16) and failure to mediate transcriptional interference (34) seen with ICI 164,384 and is consistent with the observed pure antagonist properties of this compound (ref. 17 and Fig. 2). Examples of ICI 164,384-induced receptor–DNA binding have been reported (29, 35); however, it will be interesting to determine whether this can be explained by cleavage of the 7α side group.

In summary, we have described the properties of two classes of hormone antagonists, (*i*) those that interfere with receptor–DNA binding (in the case of ICI 164,384 by inhibiting dimerization) and behave as pure antagonists and (*ii*) those such as tamoxifen and RU486 that appear to induce receptor–DNA binding but not the conformational change that activates the hormone-dependent transactivation function. It remains to be seen whether this classification can be extended to all antagonists or whether compounds can be designed to interfere directly with other receptor functions.

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