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The effect of free DNA on the interactions of the estrogen receptor bound to hormone, partial antagonist or pure antagonist with target DNA

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Interactions between the lamb uterine estrogen receptor occupied by estradiol, 4-hydroxytamoxifen (a non-steroidal partial estrogen antagonist) or ICI 164,384 (a steroidal pure estrogen antagonist), and the vitellogenin A2 estrogen-response element (vit ERE) were compared using a biotinylated 25-base all-palindromic double-stranded oligonucleotide, containing vit ERE (b-ERE), which allowed isolation of the b-ERE · receptor · [^{3}H]ligand assembly on streptavidin-Sepharose. The results of saturation analyses of the three receptor · [^{3}H]ligand complexes by increasing amounts of b-ERE were quite similar for the proportion of complexes able to interact with b-ERE (which varied from 30% to 65% according to experiments) and for the equilibrium dissociation constant [K_d(0°C) ≈1.2 nM, assuming that the receptor interacted as a dimer with b-ERE]. With each ligand, receptor binding to ERE did not change the rate of ligand dissociation from the receptor at 20°C. The rate of estrogen receptor dissociation from b-ERE, measured at 20°C in the presence of a given concentration of ERE, did not vary according to the ligand bound to the receptor; however, this dissociation rate increased linearly over the ERE concentration range (0.5–10 μM). The experimental rate constant (k_-) of estrogen receptor dissociation from b-ERE appeared to be the sum of the basal dissociation-rate constant ($k_-^0 \approx 0.011\ min^{-1}$), corresponding to spontaneous dissociation which would occur in the absence of ERE, and of the ERE-induced dissociation-rate constant, proportional to the used concentration of ERE ($k_-^i \approx 4500\ C_{ERE}\,M^{-1}\,min^{-1}$, where C_{ERE} is the molar concentration of ERE). Non-target DNA also induced receptor dissociation from b-ERE, but its efficiency was 6–10-fold lower than that of ERE. We conclude that, the two antiestrogens are as efficient as estradiol in promoting estrogen receptor binding to a single vit ERE; the low or nil ability of antiestrogens to induce estrogenic responses is probably not linked with the receptor DNA-binding step; DNA binding does not seem to affect the conformation of the filled hormone-binding site of the receptor at 20°C; interactions of receptor dimers with DNA seems to proceed by direct transfer of receptor dimers between DNA strands.

Keywords. Estrogen receptor; estrogens; antiestrogens; DNA binding; estrogen-response element.

The action of estrogens in target cells is mediated by a specific nuclear receptor. After estrogen is bound, the receptor interacts as a homodimer with target DNA usually localized in the vicinity of estrogen-regulated genes, thus modulating their transcription [1]. Most known estrogen receptor target DNAs, called estrogen-response elements (ERE), consist of short sequences that are usually not more than 13 base pairs. These ERE are identical or closely related to the 5′-GGTCAN$_1$N$_2$N$_3$TGACC sequence [2] in which the two terminal pentamers form a palindromic structure from the three central nucleotides (N, which can be any nucleotide). Such a single 13-nucleotide perfect palindromic sequence (N$_1$ = C, N$_2$, = T/A, N$_3$ = G) has been identified in the 5′ flanking region of the *Xenopus* vitellogenin A2 gene [3] whose transcription is under estrogen control [4]. This vitellogenin ERE (vit ERE) is sufficient to confer estrogen responsiveness to a heterologous promoter in gene-transfer studies [5, 6]. Moreover, mutation studies have shown that estrogen regulation of reporter gene transcription is, highly dependent upon the sequences of the two pentamers, with a very strong decrease due to a single base mutation in the canonical sequences [7], and totally abolished when the number of central nucleotides (N) is modified [8].

Various techniques have been developed to study interactions of the estrogen receptor with this vit ERE, including DNA cellulose competition [7], gel filtration [9], gel mobility-shift [10], avidin/biotin-ERE [11], and antibody-based [12] assays. The gel mobility shift assay is the most common technique. However, for quantitative determinations, the avidin/biotin-ERE assay, based on capture of the biotin-ERE · receptor · [^{3}H]ligand assembly by avidin immobilized on a matrix, is far better than the gel mobility shift assay, since it can be performed under mild conditions without modifying the equilibrium and it also provides precise and direct quantification of the free and ERE-bound receptor · ligand species. This latter technique allowed Gorski's group to determine the equilibrium dissociation constant of the rat estrogen receptor for binding vit ERE [11], and the effect of salt on the receptor/ERE interaction [13].

Synthetic antiestrogens are important therapeutic agents. Tamoxifen [14], a triphenylethylene compound, is widely used to treat estrogen-dependent breast cancer [15], while clomiphene, a related compound, is used to induce ovulation in anovulatory women [16]. Two classes of antiestrogenic ligands of the estro-

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Abbreviations. ERE, estrogen-response element; GRE, glucocorticoid-response element.

gen receptor can be distinguished, the first is constituted by partial estrogen agonists/antagonists, and includes 4-hydroxytamoxifen, a high-affinity metabolite of tamoxifen [17], as well as the two compounds mentioned above. The other, comprises pure estrogen antagonists and includes ICI 164,384 [18], a 7α derivative of estradiol. The antiestrogenic activity of these compounds could result from their inability to properly activate the estrogen receptor, as suggested by the results obtained with 4-hydroxytamoxifen [19, 20]. Estrogen receptor activation involves dimerization of the protein, dimer binding to ERE and interaction with other transcription factors. This results in transcription modulation of the corresponding estrogen-responsive genes [1, 2]. Any defect in this cascade due to defective activation of the receptor by the ligand could partially reduce or nullify the ability of the receptor · ligand complex to modulate transcription. Conflicting results have been obtained concerning the ability of antiestrogens to promote interaction of the estrogen receptor with target DNA. Fawell et al. [21] reported that the naked or 4-hydroxytamoxifen-filled estrogen receptor was able to interact with DNA, but the receptor · ICI 164,384 complex did not bind DNA since receptor dimerization was impaired. These results were not confirmed by Sabbah et al. [22] who observed that the receptor · ICI 164,384 complex bound to DNA but there were qualitative and quantitative differences with respect to the receptor · estradiol complex. Recently, Parker's group reported that the ability of ICI 164,384 and related compounds to promote receptor/DNA interactions varied considerably depending on the ligand, the type of cell expressing the receptor, the duration of receptor/ligand interactions, etc. [23]. Finally, Reese and Katzenellenbogen [24] observed that ICI 164,384 promoted receptor binding when the receptor · antiestrogen complex was formed under cell-free conditions, but not when the complex was formed in intact COS cells transfected with the estrogen receptor gene. Since all of these results were obtained using the gel-shift technique, they cannot account for the receptor/ERE interactions under equilibrium conditions. Moreover, most of the results were obtained with the receptor expressed following transfection of cells with recombinant estrogen receptor genes; the properties of these receptors could vary according to the system used and differ from those of native naturally expressed receptors [25, 26]. To more accurately compare the interactions of receptor occupied by estradiol, 4-hydroxytamoxifen or ICI 164,384 with target DNA, we therefore constructed and annealed two 25-base all palindromic complementary oligonucleotides containing vit ERE and bearing a 14-atom-arm-linked biotin residue (b-ERE) at the terminal 5′ phosphate end; then, using a native lamb uterine estrogen receptor, we performed equilibrium and kinetics studies related to the receptor/b-ERE interactions. In this study, we show that, all three receptor · ligand complexes interact with similar capacities and similar affinities with b-ERE; receptor binding to b-ERE does not increase rates of ligand dissociation from the receptor; the three complexes dissociate at identical rates from b-ERE; and this dissociation rate increases linearly with the concentration of ERE used in the dissociation experiment.

EXPERIMENTAL PROCEDURES

Materials. [6,7-^{3}H]Estradiol (specific activity 54 Ci/mmol, radiochemical purity >98%) and *Z*-4-hydroxy[*N-methyl*-^{3}H]tamoxifen (specific activity 85 Ci/mmol, radiochemical purity >95%) were purchased from Amersham International. [^{3}H]ICI 164,384 (specific activity 40 Ci/mmol, radiochemical purity ≈98%) was a gift from Dr A. E. Wakeling (ICI Pharmaceuticals Alderley Park, Macclesfield, England).

Cytosolic estrogen receptor · ligand complexes. Preparation and assay. Immature lamb uteri were stripped from connective tissue, placed in liquid nitrogen and stored at −80°C until used. The uteri were defrosted, then immediately homogenized in 4 vol. chilled 20 mM Tris/HCl pH 7.5 (T_{20}). The homogenate was centrifuged at $10^5 \times g$ for 45 min. The protein concentration of cytosol (≈5 mg protein/ml) was determined according to Layne [27]. Cytosol was supplemented with dithiothreitol (2 mM), glycerol (10%) and a cocktail of protease inhibitors: 0.2 mM phenylmethylsulfonyl fluoride, 0.4 μM aprotinin, 20 μM leupeptin and 20 μM pepstatin. Depending on experiments, cytosol was or was not diluted (to 2−5 mg protein/ml) with the same medium and supplemented with 5% dimethylformamide when [^{3}H]ICI 164,384 was used to label the estrogen receptor. Aliquots of cytosol were incubated with 5 nM [^{3}H]estradiol, 4-hydroxy[^{3}H]tamoxifen or [^{3}H]ICI 164,384 in the absence or presence of 1 μM unlabeled estradiol to measure concentrations of total bound (L_1) and non-specific bound (L_2) [^{3}H]ligands, respectively. Incubation was for 1 h at 0°C then for 30 min at 25°C to activate the receptor. After cooling at 0°C, samples were treated with an equal volume of charcoal suspension (0.5% charcoal, 0.05% dextran T70 in T_{20}) for 30 min at 0°C. The charcoal was pelleted and the radioactivity of the supernatants was measured to determine the bound concentrations L_1 and L_2 of the [^{3}H]ligand. Concentrations of receptor · [^{3}H]ligand complexes (RL) in supernatants were calculated according to Blondeau and Robel [28] from the corresponding total (L) and bound concentrations L_1 and L_2 of [^{3}H]ligands using Eqn (A) (Appendix):

$$RL = (L_1 - L_2)\frac{L}{L - L_2}. \qquad (A)$$

Proportions of non-specifically bound [^{3}H]ligands (concentration, NL) in charcoal-treated cytosol were calculated using Eqn (C) (Appendix):

$$\frac{NL}{L_1} = \frac{L_2}{L_1} \cdot \frac{L - L_1}{L - L_2}. \qquad (C)$$

Synthetic oligonucleotides. Two 25-base all palindromic oligonucleotides: 5′-GATCCAGGTCAC$^T/_A$GTGACCTGGATC and the two homologues: 5′-GATCCAGAACAC$^T/_A$GTGTTCTGGATC derived from the two formers by a four-base change (no. 8G→A; no. 9T→A; no. 17A→T; no. 18C→T), including the 13-base vit ERE and the consensus glucocorticoid-response element (GRE), respectively, were synthesized. They were then coupled at the 5′ phosphate end to a derivative of 6-aminohexanol to obtain 5′-(6-aminohexyl)oligonucleotides which were purified by ion-exchange HPLC on a Waters Gen-Pak Fax column. Biotinylated oligonucleotides were prepared by condensation of D-biotinoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester with 5′-(6-aminohexyl)oligonucleotides according to the procedure described by Agrawal et al. [29]. Briefly, 10 μmol D-biotinoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester in 1 ml dimethylformamide were added to 2 μmol 5′-(6-aminohexyl)oligonucleotide in 1 ml 0.2 M sodium carbonate, pH 9.5. After rotation of the mixture for 15 h at 20°C, biotinylated oligonucleotide was separated from the excess biotinoyl reagent on a G25 column equilibrated with T_{20}. Annealing of 5′-(6-aminohexyl)oligonucleotides and biotin-labeled oligonucleotides was performed by mixing the complementary strands in the presence of 0.2 M NaCl. The mixture was then heated to 85°C and allowed to cool to 20°C over 3 h. Aliquots of the annealed 5′-(6-aminohexyl)oligonucleotides (a-ERE and a-GRE) and biotinylated oligonucleotides (b-ERE and b-GRE) were then stored at −20°C. Concentrations of the oligonucleotide solutions were determined from absorbance at 260 nm. The absorption coefficient of each oligonucleotide was obtained by summation of the absorption

coefficients for each component and multiplied by 0.6 to correct for the loss in absorbance upon base pairing [30].

Standard DNA-binding assay. [^{3}H]Ligand-labeled, charcoal-treated cytosol (receptor · [^{3}H]ligand complex concentration: 0.5 nM < RL < 4 nM) was adjusted to 0.1 M KCl with 2.5 M KCl in T_{20} and incubated for 15 h at 0°C with 20 nM b-ERE. Aliquots (≤0.4 ml) were then incubated with 10 µl streptavidin-Sepharose (6 nmol streptavidin/ml of gel) in a final volume of 0.5 ml T_{20} containing 0.1 M KCl, 1 mM dithiothreitol, 5% glycerol and in some experiments dimethylformamide (≤2%). Samples were rotated on an orbital shaker for 1 h at 0°C to absorb biotin-linked oligonucleotide on streptavidin-Sepharose. They were then centrifuged for 5 min at $10^3 \times g$. The supernatants were discarded and the tubes were washed with 0.5 ml T_{20}. Their radioactivity was counted after extraction with 0.2 ml ethanol to determine the concentration of bound receptor · [^{3}H]ligand complex (RL_1). The concentration of non-specifically bound receptor · [^{3}H]ligand complex (RL_2) was determined by addition of 0.2 mM biotin in aliquots before incubation with streptavidin-Sepharose, to prevent interaction of the b-ERE · receptor · [^{3}H]ligand assembly with streptavidin. Specific binding of receptor · [^{3}H]ligand complex to DNA (DRL) was calculated from RL, RL_1 and RL_2 using Eqn (8) (Appendix):

$$DRL = (RL_1 - RL_2)\frac{RL}{RL - RL_2}. \quad (8)$$

Dissociation kinetics of estrogen and antiestrogens from total estrogen receptor. Aliquots of lamb uterine cytosol labeled with 5 nM [^{3}H]estradiol, 4-hydroxy[^{3}H]tamoxifen or [^{3}H]ICI 164,384, in the absence (for total binding determination) or presence of 1 µM unlabeled estradiol (for non-specific binding determination), were incubated with DNA (a-ERE or salmon sperm DNA) or vehicle. After equilibration for 30 min at 20°C, one portion of the samples was incubated in the absence of unlabeled estradiol to measure the stability of the complexes and another portion of samples was incubated in the presence of 1 µM estradiol to measure the rate of ligand dissociation from the receptor. Samples (0.3 ml) were removed at various times, chilled, then treated with charcoal to determine the concentrations of bound [^{3}H]ligand, as described for the ligand-binding assay.

Dissociation kinetics of estrogen and antiestrogens from target DNA-bound estrogen receptor. Charcoal-treated aliquots of cytosolic estrogen receptor · [^{3}H]ligand complexes, equilibrated with 20 nM b-ERE for 15 h at 0°C then for 30 min at 20°C, were incubated at 20°C in the absence of estradiol to measure the stability of receptor · [^{3}H]ligand complexes, and in the presence of 1 µM unlabeled estradiol to measure the dissociation rate of the complexes. At the times indicated, 0.2 ml samples were removed and immediately added to tubes at 0°C containing 0.3 ml T_{20} with 0.1 M KCl, 1 mM dithiothreitol, 5% glycerol and streptavidin-Sepharose, with or without biotin. Tubes were rotated for 1 h at 0°C to determine the total and non-specific binding of receptor · [^{3}H]ligand complexes, as described for the standard DNA-binding assay.

Dissociation kinetics of estrogen receptor · ligand complexes from target DNA. Charcoal-treated aliquots of cytosolic receptor · [^{3}H]ligand complexes, equilibrated with 10 nM or 20 nM b-ERE for at least 4 h at 0°C then for 30 min at 20°C, were incubated at 20°C in the absence of a-ERE, to measure the stability of the b-ERE · receptor · [^{3}H]ligand assemblies, or in the presence of various concentrations (0.5–10 µM) of a-ERE to measure the dissociation rate of the receptor · [^{3}H]ligand complexes from b-ERE. At the times indicated, 0.2 ml samples were removed and immediately added to tubes at 0°C containing 0.3 ml T_{20} with 0.1 M KCl, 1 mM dithiothreitol, 5% glycerol and streptavidin-Sepharose, with or without biotin. Tubes were rotated for 1 h at 0°C to determine the total and non-specific binding of receptor · [^{3}H]ligand complexes, as described for the standard DNA-binding assay.

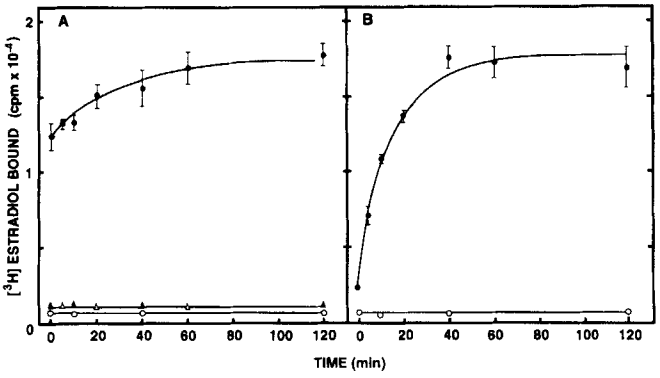

Fig. 1. Time course and specificity of estrogen receptor binding to b-ERE. [^{3}H]Estradiol-labeled, charcoal-treated cytosol (2 mg protein/ml; receptor · [^{3}H]estradiol concentration, RL = 1.6 nM) prepared as described in Experimental Procedures, was adjusted to 0.1 M KCl. (A) Aliquots (0.4 ml; 29800 cpm) of cytosol were incubated with 20 nM biotinylated ERE (b-ERE) or GRE (b-GRE) for the times indicated. They were then rotated in a final volume of 0.5 ml with streptavidin-Sepharose for 1 h at 0°C in the absence or presence of biotin for determination of total (RL_1, ● and ▲) and non-specific binding (RL_2, ○ and △) of the receptor · estradiol complex, respectively; (● and ○) incubation with b-ERE, (▲ and △) incubation with b-GRE. (B) Aliquots of the labeled cytosol fractions incubated for 4 h at 0°C with 20 nM b-ERE, were rotated with streptavidin-Sepharose for the times indicated in the absence or presence of biotin to determine total (RL_1, ●) and non-specific binding (RL_2, ○) of the receptor · estradiol complex. The amount of [^{3}H]estradiol (cpm/sample) bound to streptavidin-Sepharose in samples is represented as a function of the incubation time. Values are means of duplicate determinations.

RESULTS

Specificity of the streptavidin/b-ERE assay to measure ERE-bound ligand-labeled estrogen receptor. Using the receptor · [^{3}H]estradiol complex (Appendix) as probe, we first determined the concentration of streptavidin necessary to absorb all the biotinylated ERE (b-ERE) used in the standard assay (16 nM final concentration), and the times for receptor binding to b-ERE and for b-ERE · receptor binding to streptavidin to reach equilibrium. An approximately 30-fold excess of streptavidin (biotin-binding site concentration ≈ 0.5 µM) appeared sufficient to bind all b-ERE molecules used in the standard binding assay (data not shown). Formation of the streptavidin · b-ERE · receptor assembly at 0°C as a function of the incubation time of the receptor with b-ERE (preceding 1-h incubation with streptavidin-Sepharose) was a rapid process when the standard b-ERE concentration (20 nM) was used. For concentrations of total bound and non-specifically bound receptor, plateaus were reached within 1 h (Fig. 1 A). When lower concentrations of b-ERE (0.2 nM ≤ b-ERE ≤ 2 nM) were used, equilibrium was delayed, but it was reached within 6 h (data not shown). In all cases, binding was stable for at least 15 h. Formation of the streptavidin · b-ERE · receptor assembly as a function of the incubation time of the b-ERE · receptor entity with streptavidin-Sepharose under the standard DNA-binding assay conditions is shown in Fig. 1 B. Equilibrium was reached within 1 h incubation at 0°C and binding was stable for at least 4 h. We particularly focused on non-specific binding of the receptor · [^{3}H]estradiol complex in our assay (Appendix). Using a large excess of

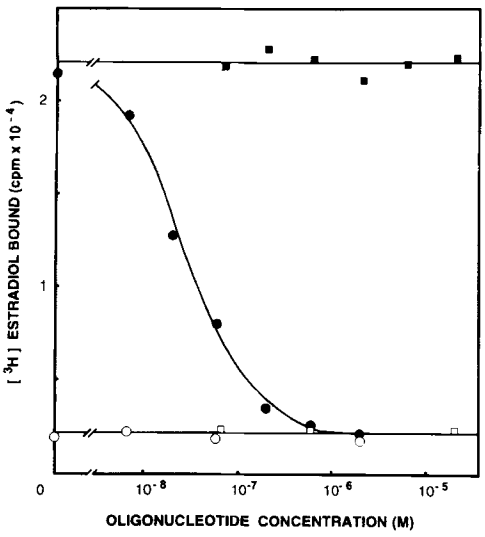

Fig. 2. Inhibition of the binding of the estrogen receptor to b-ERE by a-ERE and a-GRE. [³H]Estradiol-labeled, charcoal-treated cytosol (2 mg protein/ml; receptor · [³H]estradiol concentration, RL = 2.1 nM) prepared as described in Experimental Procedures was adjusted to 0.1 M KCl. Fractions of cytosol were incubated with 25 nM b-ERE and the indicated concentrations of the non-biotinylated oligonucleotide a-ERE (● and ○; 8.3 nM−2 μM) or a-GRE (■ and □; 83 nM−20 μM) for 15 h at 0°C. Aliquots (0.4 ml; 37900 cpm) of the cytosol fractions were rotated with streptavidin-Sepharose for 1 h at 0°C, in the absence (● and ■) or presence (○ and □) of biotin. Total (RL_1, ● and ■) and non-specific binding (RL_2, ○ and □) of the receptor · estradiol complex (cpm/sample) are represented according to the concentration of a-ERE or a-GRE. Values are means of duplicate determinations. Experimental variation was less than 10%.

biotin (0.2 mM) in the last incubation to totally prevent the b-ERE/streptavidin interaction, only one wash of the streptavidin-Sepharose gel with T_{20} was sufficient to produce a low rate (usually < 5%) of non-specific binding of the receptor · [³H]estradiol complex to the gel (Fig. 1). Two other series of experiments were performed to determine non-specific binding of the receptor · [³H]estradiol complex in our system. In the first series, the b-ERE conjugate was omitted in the first incubation; in the second series, a 100-fold excess of a-ERE, a non-biotinylated ERE, was added together with b-ERE in the first incubation. In both series of experiments, the amount of residual receptor · [³H]estradiol complex in the gel after washing was very similar to that obtained using excess biotin (data not shown). Finally, instead of b-ERE, when b-GRE, derived from b-ERE by a 2-bp change in each arm of the palindrome, was incubated with receptor · [³H]estradiol complex in the absence or presence of biotin, only low background non-specific binding (≈5%) was observed (Fig. 1A). This indicated that interaction of the receptor with b-ERE was a specific oligonucleotide sequence-dependent, and not a non-specific ionic, interaction, since b-ERE and b-GRE display the same ionic characteristics.

Characteristics of the estrogen receptor/b-ERE interaction. Gorski et al. reported that binding of the rat estrogen receptor to vit ERE only occurred with heated (1 h, 30°C) cytosol, whereas hormone did not appear to be necessary to promote DNA binding of the naked receptor [11]. They also found that binding of the receptor to target DNA was highly dependent upon the salt concentration, with optimal values at 0.1−0.15 M KCl [13]. Finally, they mentioned that up to 100% of the receptor could bind vit ERE [11]. Since our b-ERE differed at the site of biotinylation and the type of linker, from that used by Gorski's group, we

Table 1. Equilibrium dissociation constants and binding capacities of estrogen- and antiestrogen-occupied receptor for target DNA. Equilibrium dissociation constants (K_d) and maximal proportions of receptor · ligand complexes able to bind b-ERE (B_{max}, expressed as a percentage of the incubated complex) were determined from two saturation experiments of receptor · ligand complexes, using increasing concentrations of b-ERE as described in Experimental Procedures and the legend to Fig. 3. The values given are derived from binding data shown in Fig. 3 (experiment 1) and from data of another similar experiment (experiment 2).

Receptor-bound ligand	Experiment 1		Experiment 2	
	K_d	B_{max}	K_d	B_{max}
	nM	%	nM	%
Estradiol	1.23	60	1.09	46
4-Hydroxytamoxifen	1.25	51	1.19	49
ICI 164,384	1.06	50	1.24	45

determined the effects of the same parameters on the receptor/vit ERE interaction using our b-ERE. The results agreed with those already reported concerning the effects of heat, salt, and hormone. However, a difference was observed; in the presence of saturating concentrations of b-ERE and at equilibrium (incubation > 4 h at 0°C) the percentage of receptor · estradiol complex bound to b-ERE never exceeded 65% (30−65% range in the various experiments performed) in spite of the fact that protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, leupeptin and pepstatin) were added to the cytosol.

The inability of b-ERE to bind part of the cytosolic receptor estradiol complexes did not seem to result from incomplete dimerization of the complex during the activation step, since the percentage of b-ERE-bound receptor · estradiol complex did not vary according to an eightfold dilution of cytosol before the activation step (data not shown). Such a result would not be expected if the proportion of receptor (≈0.5) able to bind b-ERE reflects the proportion of dimerized receptor, which (at 0.5) would normally be closely dependent on the cytosol concentration (Appendix). Moreover, the complex not bound to b-ERE in the assay, did not bind to b-ERE in a second assay (data not shown). Finally, the addition of protease inhibitors to the cytosol only slightly improved (from 0 to 15% according to the different experiments) the percentage of the complex able to bind ERE.

Competition experiments between 25 nM b-ERE and various concentrations of a-ERE or a-GRE (derived from a-ERE by a 2-bp change in each arm of the palindrome) for binding the receptor · estradiol complex (Fig. 2), revealed a specific interaction between the complex and vit ERE, since 50% inhibition (IC_{50}) of receptor binding to b-ERE was observed at ≈ 25 nM a-ERE, whereas a-GRE (even at a 800-fold higher concentration) did not induce significant inhibition of b-ERE binding to the receptor · estradiol complex. These results indicated that biotinylation of a-ERE to obtain b-ERE did not modify the affinity of ERE for the receptor and that the affinity of ERE for the receptor was at least 1000-fold higher than that of GRE.

Receptor · estrogen and receptor · antiestrogen complexes bind with similar high affinities to b-ERE. The cytosolic lamb uterine estrogen receptor was then used to determine equilibrium dissociation constants for the interaction of estrogen-occupied or antiestrogen-occupied receptor with b-ERE. We first checked that incubation at 0°C with b-ERE, even at 0.1 μM, did not affect the measured concentrations of the various receptor · [³H]ligand complexes. Saturation experiments were then performed by incubating, a constant concentration of each of the receptor ·

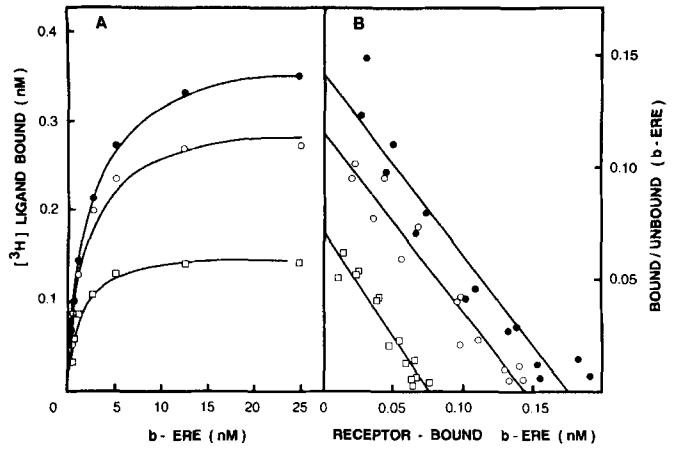

Fig. 3. Saturation analysis of b-ERE binding to estrogen-occupied and antiestrogen-occupied estrogen receptor. Cytosol (3.1 mg protein/ml) containing 5% dimethylformamide was labeled with 5 nM [^{3}H]estradiol, 4-hydroxy[^{3}H]tamoxifen or [^{3}H]ICI 164,384, then treated with charcoal as described in Experimental Procedures. Concentrations of receptor · ligand complexes (RL) in charcoal-treated cytosol fractions were 1.18, 1.14 and 0.61 nM for [^{3}H]estradiol, 4-hydroxy[^{3}H]tamoxifen and [^{3}H]ICI 164,384, respectively. Charcoal-treated fractions were diluted twofold with T_{20} containing 0.2 M KCl, 1 mM dithiothreitol and 5% glycerol. Samples were then incubated with various concentrations of b-ERE (from 0.25 nM to 25 nM) for 15 h at 0°C, then aliquots (0.4 ml) were rotated with streptavidin-Sepharose in the absence or presence of biotin for determination of total (RL_1) and non-specific binding (RL_2) of receptor · [^{3}H]ligand complexes, respectively. (A) Mean values of duplicate determinations for concentrations of specifically bound receptor · [^{3}H]ligand complexes (DRL) (calculated from RL, RL_1 and RL_2 values as described in Experimental Procedures), are plotted against the b-ERE concentration; (●) estradiol-, (○) 4-hydroxytamoxifen- and (□) ICI 164,384-occupied estrogen receptor. Experimental variation was less than 10%. (B) Scatchard plots of individual specific receptor · [^{3}H]ligand-binding data, considering that each of the receptor · [^{3}H]ligand complexes bind as a dimer to b-ERE. Regression lines were determined from plots relative to estradiol-occupied receptor ($K_A = 0.81\times10^9\ M^{-1}$, $N = 0.177$ nM (as receptor dimer), correlation coefficient 0.951), 4-hydroxytamoxifen-occupied receptor ($K_A = 0.80\times10^9\ M^{-1}$, $N = 0.145$ nM, correlation coefficient 0.967) and ICI 164,384-occupied receptor ($K_A = 0.94\times10^9\ M^{-1}$, $N = 0.0764$ nM, correlation coefficient 0.962).

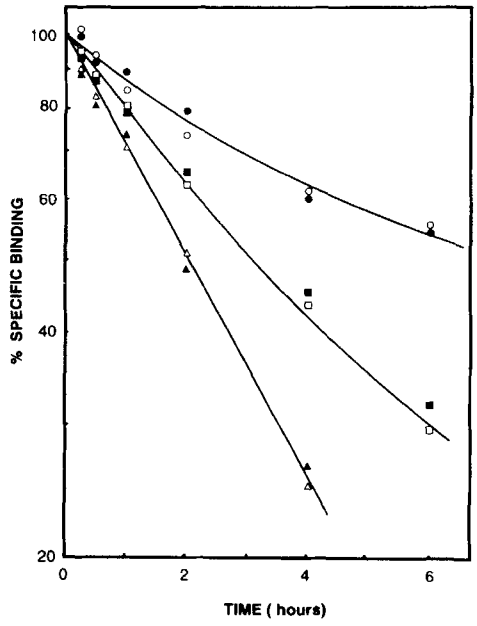

Fig. 4. Dissociation kinetics of estrogen and antiestrogens from receptor in the presence and absence of ERE. Cytosol (2 mg protein/ml) was incubated for 15 h at 0°C then for 30 min at 25°C with 5 nM [^{3}H]estradiol, 4-hydroxy[^{3}H]tamoxifen or [^{3}H]ICI 164,384 (in the latter case cytosol was supplemented with 5% dimethylformamide) in the absence or presence of 1 μM unlabeled estradiol to determine total and non-specific binding of ligands, respectively. After the samples were adjusted to 0.1 M KCl, they were incubated for 30 min at 20°C without or with 0.5 μM a-ERE. The stability and dissociation of the receptor · [^{3}H]ligand complexes at 20°C were determined as described in Experimental Procedures. Concentrations of specifically bound [^{3}H]ligands were calculated and expressed as percentages of the concentration of the [^{3}H]ligand specifically bound at time 0 (2.4, 2.3 and 1.5 nM for estradiol, 4-hydroxytamoxifen and ICI 164,384, respectively). The mean percentages from duplicate determinations for [^{3}H]ligands incubated in the presence of unlabeled estradiol are represented according to the dissociation time; (○ and ●) estradiol, (△ and ▲) 4-hydroxytamoxifen and (□ and ■) ICI 164,384. Open symbols correspond to samples incubated in the absence of a-ERE, whereas closed symbols correspond to samples incubated in the presence of a-ERE. The complexes were stable during the assay (data not shown). Experimental variation was less than 10%.

[^{3}H]ligand complexes with increasing concentrations (0.25 nM to 25 nM) of b-ERE, for 15 h at 0°C. Total and non-specific amounts of receptor · [^{3}H]ligand complexes bound to a constant amount of streptavidin-Sepharose were then measured. The saturation curves, obtained from 0.59 nM receptor · estradiol, 0.57 nM receptor · 4-hydroxytamoxifen and 0.31 nM receptor · ICI 164,384 complexes, are shown in Fig. 3A. Assuming that each of the receptor · [^{3}H]ligand complexes bound as a dimer to b-ERE, from the binding data transformed according to Scatchard [31], regression lines were determined (Fig. 3B) to obtain the binding parameters. The K_d and the B_{max} values (the latter expressed as percentages of incubated complexes) were very similar for the three complexes, ranging from 1.06 nM to 1.25 nM and from 50% to 60%, respectively (Table 1). Results obtained in another experiment (Table 1) confirmed the similarity of K_d and B_{max} values for the three complexes.

Receptor binding to DNA does not significantly modify rates of ligand dissociation from the receptor. Receptor binding to DNA was reported to increase the dissociation rate of estradiol from the receptor at 28°C [32]. To assess potential differences in the effects of DNA according to the ligand bound to the receptor, we studied the effect of both target (a-ERE) and non-target (salmon sperm) DNA on the dissociation rate of estradiol, 4-hydroxytamoxifen and ICI 164,384 from the receptor. The dissociation experiments were performed at 20°C since high dissociation rates of ligands at temperatures ≥25°C [32] make accurate determinations difficult. Dissociation rates were determined from whole populations of receptor · ligand complexes (Fig. 4), and from the fraction of complexes able to interact with b-ERE (data not shown). For whole populations of receptor · ligand complexes, and also for the sole population of complexes able to interact with b-ERE, there was no significant influence of target DNA (0.5 μM, i.e., 8 μg/ml) and non-target DNA (80 μg/ml) on dissociation rates of ligands from the estrogen receptor. Practically identical first-order processes were obtained for unbound and b-ERE-bound complexes; the dissociation-rate constants ranged from $1.8\times10^{-3}\ min^{-1}$ for estradiol to $5.2\times10^{-3}\ min^{-1}$ for 4-hydroxytamoxifen (Table 2).

Receptor · estrogen and receptor · antiestrogen complexes dissociate at identical rates from b-ERE. Since the dissociation rates of glucocorticoid and progestin receptors from target DNA were reported to be markedly but differently modified by hormone and antihormone binding to the receptor [33], we compared the kinetics of estrogen receptor dissociation from b-ERE

Table 2. Kinetic dissociation-rate constants. Kinetic rate constants for dissociation of ligands from receptor at 20°C were determined (a) from total receptor · ligand complexes previously incubated without (Control) or with 0.5 μM a-ERE (DNA) as described in Experimental Procedures and in the Fig. 4 legend and (b) from the fraction of receptor · ligand complexes bound to b-ERE in the absence (control) or in the presence of 80 μg salmon sperm DNA/ml (DNA) as described in Experimental Procedures. Kinetic rate constants for dissociation of receptor · ligand complexes from b-ERE at 20°C were determined from complexes equilibrated with 10 nM b-ERE in the presence of 0.5 μM a-ERE, as described in Experimental Procedures and in the legend to Fig. 5. All values are means of two independent determinations.

Dissociation of		Bound-ligand	Dissociation-rate constant (k_-)	
			control	DNA
			$min^{-1} \times 10^3$	
R · L	(a) from total receptor	estradiol	1.89 ± 0.25	1.88 ± 0.32
		4-hydroxytamoxifen	5.22 ± 0.44	5.48 ± 0.61
		ICI 164,384	3.50 ± 0.30	3.18 ± 0.40
	(b) from b-ERE-bound receptor	estradiol	1.78 ± 0.14	1.77 ± 0.18
		4-hydroxytamoxifen	5.10 ± 0.48	5.05 ± 0.43
b-ERE · R		estradiol	11.8 ± 0.9	
		4-hydroxytamoxifen	11.3 ± 1.1	
		ICI 164,384	12.1 ± 1.0	

according to the ligand bound to the receptor. That high concentrations of a-ERE or non-target DNA did not increase the rate of ligand dissociation from receptor (Fig. 4, Table 2), allowed us to determine the dissociation rate of the various receptor · ligand complexes from b-ERE (10 nM) at 20°C, in the presence of a large excess of a-ERE (0.5 μM) to prevent dissociated [^{3}H]ligand-labeled receptor from reassociating with b-ERE. In the absence of a-ERE, the amount of ligands specifically adsorbed on streptavidin did not vary according to time (Fig. 5), indicating that there was no denaturation or loss of b-ERE · receptor · ligand assemblies during the assay. In the presence of a-ERE, the amount of specifically adsorbed ligands on streptavidin decreased over time according to first-order processes. There was no significant difference in the dissociation kinetics of the receptor from b-ERE according to the ligand, estradiol, 4-hydroxytamoxifen or ICI 164,384 bound to the receptor. In the three cases, we determined very similar rate constants (Table 2).

DNA increases the dissociation rate of receptor · ligand complexes from b-ERE. The last point studied was related to the potential effect of target and non-target DNA on the dissociation rate of complexes from b-ERE. Experiments similar to those described above were performed using increasing concentrations of a-ERE (≥0.5 μM). As illustrated in Fig. 6 with the receptor · estradiol complex, quasi-first-order processes were observed for dissociation of the three complexes from b-ERE, at the various concentrations of a-ERE used (from 0.5 μM to 10 μM in the various experiments). However, the dissociation rate (identical for the three complexes) greatly depended upon the concentration of a-ERE used in the assay. A quasi linear relation was noted between the measured dissociation rate and the a-ERE concentration used. Extrapolation to 0 concentration of a-ERE gave a value of 0.011 min^{-1} for k_-. On the basis of these results, the rate constant k_- of receptor dissociation from b-ERE appeared to be the sum of two components: $k_- = k_-^\circ + k_-^i$; where k_-° is the basal dissociation-rate constant which would be obtained in the absence of a-ERE, and k_-^i is the a-ERE-induced dissociation-rate constant directly proportional to the concentration of a-ERE used. At 20°C, k_-° = 0.011 min^{-1} and k_-^i = 4500 C_{ERE} $M^{-1}min^{-1}$, where C_{ERE} is the molar concentration of a-ERE. The induced dissociation of receptor · ligand complexes from b-ERE was not restricted to target DNA. Using a constant concentration (0.5 μM) of a-ERE to prevent dissociated receptor from reassociating with b-ERE, and increasing concentrations of non-target DNA (either salmon sperm DNA or Gal4-target 17-base oligonucleotide), we observed similar processes (data not shown). However, the potency of these non-target DNAs was 6–10-fold lower than that of a-ERE.

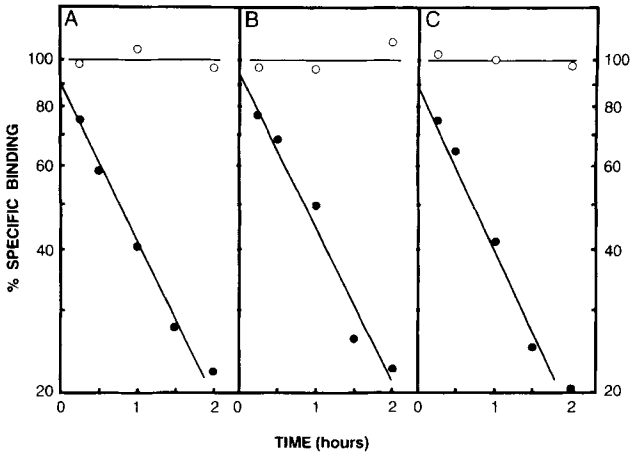

Fig. 5. Dissociation of estrogen-occupied and antiestrogen-occupied estrogen receptor from b-ERE. Cytosol (4 mg protein/ml) containing 5% dimethylformamide was labeled with 5 nM [^{3}H]estradiol, 4-hydroxy[^{3}H]tamoxifen or [^{3}H]ICI 164, 384 then treated with charcoal as described in Experimental Procedures. Concentrations of receptor · ligand complexes (RL) in charcoal-treated cytosol fractions were: 3.3 nM, 3.1 nM and 2.2 nM for [^{3}H]estradiol, 4-hydroxy[^{3}H]tamoxifen and [^{3}H]ICI 164,384, respectively. Charcoal-treated fractions supplemented with KCl (0.1 M final concentration) were incubated with 10 nM b-ERE for 15 h at 0°C then for 30 min at 20°C. Samples were then incubated at 20°C in the absence (to measure stability of b-ERE · receptor · [^{3}H]ligand assemblies) or presence of 0.5 μM a-ERE (to measure dissociation of receptor · [^{3}H]ligand complexes from b-ERE). The dissociation (●) and stability (○) of b-ERE-bound receptor · estradiol (A), receptor · 4-hydroxytamoxifen (B) and receptor · ICI 164,384 (C) complexes were determined by a standard DNA-binding assay. The mean specific binding of receptor · [^{3}H]ligand complexes from duplicate determinations is represented according to the incubation time, as a percentage of the corresponding undissociated b-ERE · receptor · [^{3}H]ligand assembly. Experimental variation was less than 15%.

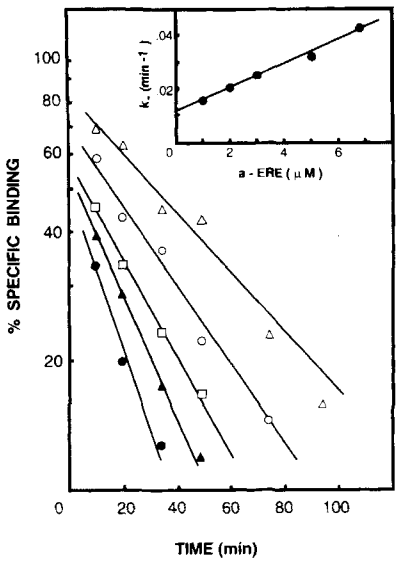

Fig. 6. Effect of a-ERE on the dissociation rate of the receptor · estradiol complex from b-ERE. [^{3}H]Estradiol-labeled, charcoal-treated cytosol (2 mg protein/ml; receptor · [^{3}H]estradiol concentration, RL = 2.1 nM) was supplemented with KCl (0.1 M final concentration) and incubated with 20 nM b-ERE for 4 h at 0°C then 30 min at 20°C. Aliquots of cytosol were incubated at 20°C in the absence (to measure the stability of the b-ERE · receptor · [^{3}H]estradiol assembly) or presence of various concentrations (1−6.75 μM) of a-ERE (to measure dissociation of the receptor · [^{3}H]estradiol complex from b-ERE). The stability and dissociation of the b-ERE · receptor assembly were determined by a standard DNA-binding assay. The specific binding of receptor · [^{3}H]estradiol complex (mean of duplicate determinations) in the presence of the various concentrations of a-ERE is represented according to the incubation time, as a percentage of undissociated b-ERE · receptor · [^{3}H]ligand assembly (which is not shown, but did not vary according to time, as in Fig. 5); (△) 1 μM a-ERE, (○) 2 μM a-ERE, (□) 3 μM a-ERE, (▲) 5 μM a-ERE, (●) 6.75 μM a-ERE. Experimental variation was less than 15%. Variation of the dissociation rate calculated from dissociation curves, according to the a-ERE concentration, is shown in the inset.

DISCUSSION

The results of these studies directly demonstrated specific interactions of lamb estrogen receptor · [^{3}H]ligand complexes, activated under cell-free conditions, with a biotinylated 25-bp oligonucleotide containing vit ERE. The b-ERE/avidin system was used to determine the kinetic and equilibrium parameters related to interactions of the receptor occupied by hormone, partial agonist/antagonist or pure antagonist, with ERE. Several results differed from those reported by Gorski et al. for interactions of the rat cytosolic estrogen receptor · estradiol complex with another type of biotinylated vit ERE [11, 13]. The values we determined for the percentage of complexes able to interact with vit ERE and for the equilibrium affinity constant relative to the receptor/vit ERE interaction were lower than those determined by Gorski et al. (percentage of receptor able to interact with vit ERE; 30−65% versus 60−100%; equilibrium association constant, approximately 0.8×10^9 M^{-1} versus 3 to $6\times10^9 M^{-1}$). Moreover, we did not observe any significant difference in the dissociation rate of estradiol or the two antiestrogens from the receptor at 20°C according to the state of the receptor, unbound or bound to target or non-target DNA; whereas Gorski's group found that target DNA increased the dissociation rate of estradiol and estriol from the estrogen receptor and concluded that DNA allosterically modulates the steroid-binding domain of the estrogen receptor [32].

Experiments using decreasing receptor concentrations indicated that the inability of part of receptor · ligand complexes to interact with vit ERE did not result from incomplete dimerization of a homogeneous population of receptor monomers; instead they suggested the presence of two receptor populations in tissue extracts. The presence of these two populations could reflect either true heterogeneity in receptor molecules or the presence, in the cytosol, of a limited amount of a factor required to promote receptor binding to target DNA [34].

One plausible explanation for the discrepancy in the K_A values determined in this study as compared to those reported by Gorski et al. could be that our determinations were carried out assuming that receptors interacted as homodimers with the b-ERE duplex (stoichiometry b-ERE/receptor, 1:2), whereas those of Gorski et al. were carried out assuming a 1:1 stoichiometry. In case of a high-affinity interaction, the bound/free ratio relative to one of the interacting species (i.e., b-ERE) and then K_A could greatly decrease when calculated assuming 1:2 stoichiometry rather than 1:1 stoichiometry. In any case, the 0.8×10^9 M^{-1} value we determined for K_A at 0°C was consistent with the stability of the b-ERE · receptor assembly whose half-dissociation time at 20°C was approximately 1 h, since both the affinity (at 0°C) and the half-dissociation time (at 20°C) for the receptor/estradiol interaction are known to be about 10-fold higher [35]. In the cellular context, due to cooperative interactions between receptor dimers bound to distinct ERE or between one receptor dimer bound to ERE and other transcription factors bound to their target DNAs, the resulting affinity of the estrogen receptor for the target gene promoter could be much higher than that determined in cell-free systems using a single ERE.

The characteristics of the receptor/ERE interaction did not vary appreciably according to the ligand, estradiol, partial antagonist or pure antagonist bound to the receptor. The percentages of receptor molecules able to bind ERE, the equilibrium dissociation constants at 0°C and the kinetic dissociation-rate constants at 20°C were practically identical in all three cases. Using an indirect antibody-based DNA-binding assay which seems to be less reliable than the b-ERE/avidin DNA-binding assay (since receptor · ligand complexes could be dissociated by receptor antibodies [36]), Gorski et al. [12] concluded that there was no significant variation in both the capacity of receptor to bind ERE and the equilibrium affinity constant, according to the ligand bound to the estrogen receptor. These identical characteristics for estrogen-liganded and antiestrogen-liganded estrogen receptors strongly suggest that receptor · antiestrogen complexes are as efficient as receptor · estrogen complexes in interacting with ERE, and that antiestrogenic effects probably result from impaired action of these compounds at step(s) other than the interaction of the receptor with ERE, e.g., down-regulation of the receptor [37, 38], or defective interaction of the receptor · antiestrogen complexes with other transcription factors.

The identical rates of dissociation of receptor · estrogen and receptor · antiestrogen complexes from b-ERE at 20°C found in this study, contrasted with the marked difference in the dissociation rates at 25°C from target and non-target DNA reported for both glucocorticoid and progesterone receptors [33], according to the ligand, hormone or antihormone bound to the receptor. In these cases, the authors reported that the kinetic dissociation rates of receptor · antihormone complexes from DNA were lower than those of steroid-free receptors but higher than those of hormone-filled receptors. However, with these fast dissociating ligands at 25°C, a trivial factor, i.e. difference in stabilities of the receptor · hormone and the receptor · antihormone complexes (not checked in these experiments) could account for the observed effects.

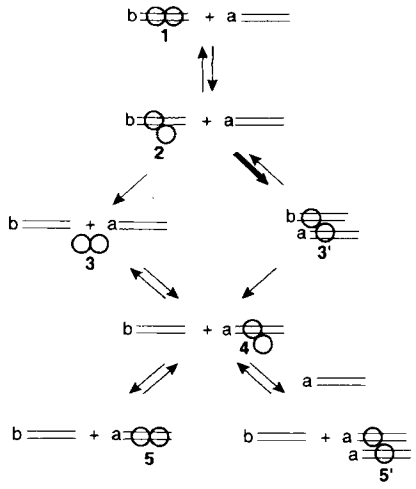

Fig. 7. Model for ERE-induced dissociation of the estrogen receptor from ERE. A two-step process for dissociation of receptor dimer from b-ERE, which can account for a-ERE-induced dissociation is schematically represented. To show dissociation of the receptor dimer · b-ERE complex (form 1), a-ERE (in large excess over b-ERE) is initially added to the complex. In a first reversible step (form 1 ⇌ form 2), one molecule of the receptor in the dimer partially or totally dissociates from its binding site on b-ERE, while the second receptor molecule is still bound to the second half-binding site on b-ERE. Spontaneous dissociation of the receptor dimer from b-ERE (left side of the scheme) is achieved in a second step when the second receptor molecule dissociates from b-ERE (form 2 → form 3); then a-ERE totally prevents reassociation of the receptor dimer with b-ERE, making this second step irreversible and leads to form 4 and form 5. In this model, at any stage of the process, the receptor dimer bound only one target DNA and a-ERE plays only a passive role. a-ERE-induced dissociation (right side of the scheme) could also take place in the process from form 2. It is conceivable that the receptor molecule dissociated (partially or totally) in form 2, could interact with a-ERE to give a transitory heteromeric complex 3′ in which one receptor dimer bound both a half-binding site of b-ERE and a half-binding site of a-ERE. Form 3′ either comes back to form 2 or irreversibly evolves towards form 4, then towards forms 5 and 5′. If we assume that stability of 3′ is lower than that of form 2, then dissociation of the receptor dimer from target DNA in the presence a-ERE will be higher than in its absence. Relative formation of 3′ from 2 could be directly proportional to the a-ERE concentration. Hence the linear relationship between the concentration of a-ERE used and the dissociation rate of the receptor from b-ERE. In this last model, a-ERE plays an active and direct role in the dissociation process.

Another interesting result of this study was the dissociation of complexes from ERE induced by micromolar concentrations of target or non-target (with a lower efficiency) DNA. The induced dissociation rate appeared to be directly proportional to the DNA concentration. Fig. 7 illustrates one possible mechanism for a-ERE-induced dissociation of the receptor dimer from b-ERE, in the case of two-step dissociation. In such a case, the induced dissociation rate would be proportional to the concentration of a-ERE. The lower efficiency of non-target DNA to induce dissociation of the receptor dimer from b-ERE could result from its low affinity for the receptor relative to that of b-ERE. However, when the receptor dimer is bound to non-target DNA instead of ERE, then non-target DNA could be more efficient in inducing dissociation of the dimer. Such induced dissociation could be involved in efficient searching for target DNA by the receptor. Our results suggest that direct transfer of receptor dimers between DNA strands (coupled or not with sliding of bound dimers along DNA), may occur in the nucleus of target cell for rapid encounters between target DNAs and receptor dimers. This model would be more plausible than the classical cyclic model (repeated cycles of association and dissociation of receptor dimers with and from DNA, until a high-affinity site is found) [39]. The high concentration of DNA in the cell nucleus (≈13 mg/ml, assuming a radius of 5 nm for a nucleus of the estrogen-target cell) is compatible with this model. From total DNA, the theoretical concentration of non-specific binding sites (15 base pairs) for receptor dimers would be approximately 1.3 mM. Even when only 1% of total DNA is available to the receptor in non-cycling cells, the concentration of useful DNA in the transfer model would be higher than 10 μM, a concentration which was found to be very efficient for inducing dissociation of DNA-bound receptors in the cell-free system used.

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APPENDIX

Correction for non-specific binding in the determination of receptor · ligand complexes. The concentration of the receptor · [^{3}H]ligand complex (RL) formed on incubation of cytosol with [^{3}H]ligand is higher than the difference between bound concentrations of [^{3}H]ligand (L_1 and L_2) measured from cytosol incubated with the [^{3}H]ligand, in the absence or presence of excess unlabeled estradiol, respectively. Since the receptor is occupied by unlabeled estradiol, the concentration of [^{3}H]ligand available for non-specific binding is higher in the presence than in absence of unlabeled estradiol. Therefore, L_1 includes a concentration of non-specifically bound [^{3}H]ligand (NL) lower than L_2. Assuming that the saturable binding of [^{3}H]ligand reflects formation of the receptor · [^{3}H]ligand complex and that L_2 is strictly proportional to the concentration of [^{3}H]ligand incubated (L), the correct value for RL, established by Blondeau and Robel [28], is

$$RL = (L_1 - L_2)\frac{L}{L - L_2}. \quad (A)$$

Note that

$$NL = L_1 - RL = \frac{L_2(L - L_1)}{L - L_2}. \quad (B)$$

The proportion of non-specifically bound [^{3}H]ligand in charcoal-treated cytosol is then

$$\frac{NL}{L_1} = \frac{L_2}{L_1} \cdot \frac{L - L_1}{L - L_2}. \quad (C)$$

With ligands such as estradiol, that display high affinity for the receptor and relatively low hydrophobicity, it is possible to saturate a high proportion of the cytosolic receptors without producing a high rate of non-specific binding. The use of a [^{3}H]estradiol concentration approximately twofold higher than the receptor concentration, saturated more than 95% of the receptor molecules, usually with a very low proportion of non-specifically bound [^{3}H]estradiol in charcoal-treated cytosol ($NL/L_1 < 0.03$). Therefore, the concentration of total bound [^{3}H]estradiol after charcoal treatment could be equated to the concentration of the receptor · [^{3}H]estradiol complex ($RL < L_1 < 1.03$ RL). Using 4-hydroxy[^{3}H]tamoxifen under the same conditions, a similar level of receptor occupancy (>95%) was obtained. However, due to the hydrophobicity of the ligand, the rate of non-specific binding ($NL/L_1 \approx 0.1$) was higher than that of [^{3}H]estradiol. Here, L_1 could not be equated to the concentration of the receptor · 4-hydroxy[^{3}H]tamoxifen complex ($L_1 \approx 1.11$

RL) and Eqn (A) must be used to determine the actual complex concentration. With the highly hydrophobic ligand ICI 164,384 [18], formation of the receptor · [^{3}H]ICI 164,384 complex was a less favorable process than in the case of estradiol or 4-hydroxytamoxifen. To obtain a fair proportion of receptor occupancy with this ligand together with a reasonable level of non-specific binding, we determined the concentrations of specifically bound and non-specifically bound [^{3}H]ICI 164,384 in cytosol according to both the concentration of the compound (1–10 nM) and the proportion (0–10%) of dimethylformamide, a solvent which decreases hydrophobic interactions. Satisfactory results, i.e. receptor occupancy greater than 50% with reasonable non-specific binding ($NL/L_1 \approx 0.2-0.3$) were obtained when receptor-rich cytosol (1–2 pmol receptor/mg protein) was incubated in the presence of 5% dimethylformamide with an [^{3}H]ICI 164,384 concentration approximately twofold higher than the receptor concentration. The high values obtained for the L_1/RL ratio ($L_1 \approx 1.25-1.45$ RL, according to experiments) still required the use of Eqn (A) to determine the actual concentration of the receptor · [^{3}H]ICI 164,384 complex.

Correction for non-specific binding in the determination of the b-ERE · receptor · ligand assembly. On the basis of the ligand and b-ERE binding experiments, it appears that the uterine cytosol includes two populations (R_A and R_B) of estrogen receptors. Both populations bind estrogens and antiestrogens but only R_A is able to bind b-ERE. In the DNA-binding assay, the two populations also interact non-specifically (low-affinity and non-saturable binding) with streptavidin-Sepharose. When a receptor · [^{3}H]ligand complex (RL) is incubated with b-ERE the distribution of the complex can be represented as

$$RL = R_AL + R_BL = DR_AL + UR_AL + R_BL \quad (1)$$

where R_AL and R_BL are the total concentrations of the two forms of receptor · ligand complex, and DR_AL and UR_AL are the concentrations of specifically target DNA-bound and unbound R_AL complexes, respectively. Assuming that the whole b-ERE-bound complex is specifically retained, and only fractions f_A and f_B of unbound complexes R_AL and R_BL are non-specifically retained on streptavidin-Sepharose, the measured concentration of complex in the gel is

$$\begin{aligned} RL_1 &= DR_AL + f_A \cdot UR_AL + f_B \cdot R_BL \\ &= DR_AL + f_A(R_AL - DR_AL) + f_B \cdot R_BL. \end{aligned} \quad (2)$$

Three methods can be used to determine non-specific binding of R_AL and R_BL complexes to streptavidin-Sepharose. One possibility is to omit b-ERE in the assay, then all the R_AL complexes are available for non-specific binding. Another possibility is to use a large excess of non-biotinylated ERE (a-ERE) together with b-ERE to prevent binding of R_AL complexes to b-ERE. The third possibility is to add a large excess of biotin in the last incubation to prevent binding of b-ERE-bound complexes to streptavidin-Sepharose. In the last two cases, if we admit that a-ERE-bound (second case) and b-ERE-bound (third case) complexes behave as unbound R_AL complex for non-specific binding to streptavidin-Sepharose, the measured concentration of complex in the gel by each of the three methods is

$$RL_2 = f_A \cdot R_AL + f_B \cdot R_BL. \quad (3)$$

Subtraction of Eqn (3) from Eqn (2) gives

$$RL_1 - RL_2 = DR_AL\,(1 - f_A). \quad (4)$$

Therefore

$$DR_AL = (RL_1 - RL_2)\frac{1}{1 - f_A}. \quad (5)$$

If we assume that $f_B \approx f_A$, Eqn (3) becomes

$$RL_2 \approx f_A\,(R_AL + R_BL) = f_A \cdot RL. \quad (6)$$

Then

$$f_A = \frac{RL_2}{RL}. \quad (7)$$

From Eqns (5 and 7) the expression of DR_AL becomes

$$DR_AL = (RL_1 - RL_2)\frac{RL}{RL - RL_2}. \quad (8)$$

The concentration of complex specifically bound to b-ERE can be calculated from concentrations of total incubated (RL), total bound (RL_1) and non-specifically bound (RL_2) complex.

Receptor dimerization and binding to b-ERE. In case of a single dimerization process for the receptor · ligand complex occurring during the activation step

$$2RL \rightleftharpoons (RL)_2.$$

Assuming that the formation of the dimer is a second-order process relative to the monomer concentration, then at equilibrium

$$\frac{(RL)_2}{RL^2} = K_2 \quad (\alpha)$$

with:

$$2(RL)_2 + RL = N \quad (\beta)$$

where RL, $(RL)_2$ and N are the concentrations of receptor monomer, receptor dimer and total receptor concentration (evaluated as monomer), respectively, and K_2 is the dimerization constant.

From Eqns (α) and (β) it becomes

$$(RL)_2 = \frac{N}{2} + \frac{1}{8K_2}\left(1 - \sqrt{8K_2N + 1}\right). \quad (\gamma)$$

Therefore, the proportion of dimerized receptor is

$$p = \frac{2(RL)_2}{N} = 1 + \frac{1}{4K_2N}\left(1 - \sqrt{8K_2N + 1}\right). \quad (\delta)$$

Conversely from experimental values of N and p, using Eqn (δ) it is possible to determine K_2. Taking, for undiluted cytosol, $N = 1$ nM and $p = 0.5$ (assuming that the proportion of dimerized receptor · ligand complexes is the same that the proportion of complexes able to interact with b-ERE), it becomes $K_2 = 10^9\ M^{-1}$. This value can be used to determine the effect of cytosol dilution on the value of p; for instance an eightfold dilution of cytosol would result in $p \approx 0.17$, 2.9-fold lower than the value corresponding to undiluted cytosol. The fact that such a cytosol dilution had no effect on the proportion of receptors able to interact with b-ERE suggests that, incomplete dimerization of homogeneous receptor monomers is not the cause of the inability of part of the receptor molecules to interact with b-ERE, and for receptor monomers able to dimerize then interact with b-ERE, $K_2N \gg 1$, i.e., $K_2 \gg 10^9\ M^{-1}$.

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