Estrogen Receptor Ligands Modulate Its Interaction with DNA*

Boris J. Cheski§, Sotirios Karathanasis, and C. Richard Lytle
From the Women’s Health Research Institute, Wyeth-Ayerst Research, Radnor, Pennsylvania 19087

The estrogen receptor (ER) belongs to a superfamily of ligand-inducible transcription factors. Functions of these proteins (dimerization, DNA binding, and interaction with other transcription factors) are modulated by binding of their corresponding ligands. It is, however, controversial whether various ER ligands affect the receptor’s ability to bind its specific DNA element (ERE).

By using real-time interaction analysis we have investigated the kinetics of human (h)ER binding to DNA in the absence and presence of 17β-estradiol, 17α-ethynyl estradiol, analogs of tamoxifen, raloxifene, and ICI-182,780. We show that ligand binding dramatically influences the kinetics of hER interaction with specific DNA. We have found that binding of estradiol induces the rapid formation of a relatively unstable ER-ERE complex, and binding of ICI-182,780 leads to slow formation ($k_d$ is approximately 10 times lower) of a stable receptor-DNA complex ($k_f$ is almost 2 orders of magnitude lower). Therefore, binding of estradiol accelerates the frequency of receptor-DNA complex formation more than 50-fold, compared with unliganded ER, and more than 1000-fold compared with ER liganded with ICI-182,780. We hypothesize that a correlation exists between the rate of gene transcription and the frequency of receptor-DNA complex formation. We further show that a good correlation exists between the kinetics of hER-ERE interaction induced by a ligand and its biological effect.

Steroid hormones are widely distributed small, lipophilic molecules that participate in intracellular communication and control a wide spectrum of developmental and physiological processes. Their effects are mediated by specific intracellular receptors, a family of proteins that are characterized by a high affinity for the corresponding hormones and an ability to discriminate between structurally closely related ligands. These ligand-inducible receptors can modulate transcription of target genes by virtue of their binding to a specific sequence on DNA in target promoters known as hormone response elements (1–4).

Binding of 17β-estradiol (E$_2$) to estrogen receptor (ER) is followed by a conformational change, leading to dissociation of the receptor from the complex with the heat shock proteins hsp90 and p59 (5, 6), dimerization (7, 8), and activation of DNA binding. After DNA binding the activated receptor can interact with basal transcription factors (9). These interactions are thought to stabilize the preinitiation complex at the promoter, allowing RNA polymerase to initiate transcription (10). Recently a number of transcriptional intermediary factors have been identified that can modify estrogen responsiveness, and several of these proteins interact with the ligand binding domain of the ER in a ligand-dependent manner (11–13).

It is obvious that the ligand plays a key role in initiating this cascade of events. However, it remains controversial as to whether estrogen affects the receptor’s ability to bind specific DNA (ERE). Initially, in vitro analysis performed with hER (HEO) expressed in HeLa cells, Xenopus oocytes, yeast, or produced in in vitro transcription/translation indicated that binding of E$_2$ to an ERE was hormone-dependent and that E$_2$ induced the formation of receptor dimers (8). It was subsequently found that this protein (HEO) has an artificial mutation (Gly-400 → Val) that decreases hormone binding at 25 °C but not at 4 °C (14).

Subsequently it was reported that wild type hER (HEGO) binds DNA in absence of ligand (15, 16). Hormone-independent formation of ER-ERE complex was also reported with crude extracts (17) and purified ER from calf uterus (18), rat uterine extracts (19), mouse uterine extracts (20), transfected COS-1 cells (21, 22), and S9 cells infected with recombinant baculovirus (21). In contrast, ligand-induced DNA binding was reported with HEGO, produced by in vitro transcription/translation and in S9 cells (23). In vivo ligand-dependent and ligand-independent ERE association was reported for hER and Xenopus ER using promoter interference assay (24, 25). It was also shown that hormone may be required to promote DNA binding at low but not at high concentrations of ER (26). Genomic footprinting indicated that occupation of the ERE present in apoVLDLII promoter region is hormone-dependent (27) which suggests that the hormone is affecting ER interaction with ERE.

We hypothesized that ligand binding may affect the kinetics of ER interaction with DNA while having minimal effect on its affinity. It was previously reported that binding of hormone accelerates the kinetics of glucocorticoid and progesterone receptor binding to DNA (33), affects dimerization status and the kinetics of DNA binding of vitamin D$_3$ receptor (28). In this case the discrepancies reported previously with the ligand effect on ER-ERE interactions may be due to the fact that the methodologies used could not detect the dynamics of the protein-DNA complex formation.

We have examined the ER-ERE interactions using surface plasmon resonance (SPR) methodology. Using this approach, from mouse complement component C3 gene promoter; GSA, gel shift assays. SPR, surface plasmon resonance; bp, base pair(s); RU, resonance units; FC, flow cell(s).
we have found that ligand binding dramatically affects the kinetics of hER interaction with specific DNA. We show that binding of agonists or antagonists by hER has opposing effects on the kinetics of ER binding to ERE.

EXPERIMENTAL PROCEDURES

Equipment and Reagents—The BIAcore system, sensor chips CM 5 (certified), Twente 20, the amine coupling kit containing N-hydroxysuccinimide, N-ethyl-N-(3-dimethylaminopropyl) carbodiimide and ethanolamine hydrochloride were all obtained from Biacore Inc. The buffer uses for all experiments was 50 mM Tris-HCl, 150 mM NaCl, 0.05 mM MgCl2, 0.05% Tween 20, pH 7.5. 17β-Estradiol, 17α-ethynyl estradiol, 4-hydroxytamoxifen, and tamoxifen were obtained from Sigma; 3-hydroxysteroids was obtained from RBL; raloxifene was synthesized by Wyeth-Ayerst Medicinal Chemistry group. ICI-182,780 was provided by Zeneca Pharmaceuticals. [3H]-17β-Estradiol was from DuPont NEN.

Bioconjugation of Oligonucleotide—Several potential EREs were synthesized as self-anneling oligonucleotides that form hairpin duplex upon heating and rapid cooling. A 75-2p oligonucleotide (Vit.A2) containing a specific binding site for ER was derived from the vitellogenin A2 gene of Xenopus laevis response promoter. Its sequence is 5′-AGCTTCTTGTACGTCATCAGCTGACCTGAACTTACCCCCGAGCAA-3′. The second oligonucleotide (C3) was derived from mouse complement component C3 gene promoter (29). Its sequence is 5′-AGCTCTTGTACCCTGACCTGAACTTACCCCCGAGCAA-3′. The third oligonucleotide (C3) was derived from mouse complement component C3 gene promoter (29). Its sequence is 5′-AGCTCTTGTACCCTGACCTGAACTTACCCCCGAGCAA-3′. Following annealing, the oligonucleotides were biotinylated by incorporation of biotin-dATP with Klenow enzyme and purified from unincorporated biotinylated nucleotides by gel filtration on a Chromaspin 10 column (CLONTECH).

Preparation of the Sensor Chip—The BIAcore® biosensor system (Pharmacia Biotech, Upssala, Sweden) permits the monitoring of macromolecular interactions in real time. The detection system uses SPR, a quantum mechanical phenomenon that detects changes in the refractive index at the surface of sensor chip (30). The binding of a soluble ligand to the immobilized one leads to an increase in the ligand concentration at the sensor surface, with a corresponding increase in the refractive index. This refractive index change alters the SPR which can be detected optically (40). BIAcore 2000 allows the simultaneously detection of the interaction events on four different spots, located in different flow cells (FC), on the sensor surface. The flow system allows the sample to be addressed to individual FCs or predefined combinations of the four FCs. To immobilize specific DNA, the surface of a CM 5 sensor chip (certified) was first modified with streptavidin according to instructions from the manufacturer. The surface was activated by injection of a solution containing 0.2 M N-ethyl-N-(3-dimethylaminopropyl) carbodiimide and 0.05 M hydroxysuccinimide (HSS) for four different surfaces 2918, 1922, 1363, and 908 resonance units (RU) of streptavidin were obtained by injecting streptavidin at 10 μg/ml and varying a contact time from 1 to 6 min. Gradient surfaces with 1730, 1300, 1000, and 600 RU of Vit.A2-DNA were then obtained by injection of 50 μM of biotinylated oligonucleotide solution at 33 ng/ml. After each protein injection, the surface was regenerated with one 10-μl injection of 0.1% SDS solution. One cycle of regeneration was sufficient to remove bound ER.

Binding Assay and Data Analysis—Each binding cycle was performed with a constant flow of 50 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl2, 0.05% Tween 20, pH 7.5, buffer of 5 μM/min. Samples of ER were injected across the surface via a sample loop. Once the injection was complete, the formed complexes were washed with the buffer for an additional 500–1000 s. All experiments were performed at 25 °C. Data were collected at 1 Hz and analyzed using the BIAEvaluaction program 2.1 (Biacore Inc) on a Compaq PC. This program uses a nonlinear least squares analysis method for the determination of rate binding constants for macromolecular interactions. The dissociation kinetics of the ER:ERE complexes can be described by a double exponential decay as follows: A(t) = A0 - A + B. One of these two components disappears from the surface with apparent rate of h0.5 = 0.01 s-1 which corresponds to a complex half-life of several minutes and represents only 5–10% of total bound protein. The second complex is much more stable. Its apparent dissociation rate h1.2 = 10-7 to 10-8 s-1, depending on the nature of ligand, and it represents approximately 90–95% of total bound protein. Association kinetics can be described by a model that uses two analytes (A1 and A2) interacting with the same binding site as follows: A1 + A2 + B = A1B + A2B (28).

The apparent equilibrium dissociation constants for the second complex, which represent the majority of bound material, were calculated from the two rate constants (Kd = k2/k1). Purification of hER—Partially purified recombinant human estrogen receptor was obtained from an E. coli expression system (about 80% pure) and purified to homogeneity as assessed by visualization on a Coomassie-stained SDS gel by gel filtration on Superdex-200 column (Pharmacia) and chromatography on Ni2+ affinity column (Pharmacia). Receptor was eluted from Ni2+ affinity column with a linear gradient of imidazole from 0 to 0.5 M in 50 mM Tris-HCl, 500 mM NaCl, pH 7.5.

Gel Filtration Chromatography—A Superdex 200 (30.10) column was obtained from Pharmacia. The column was equilibrated with 50 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl2. 0.05% Tween 20, pH 7.5, and calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and RNase (13.7 kDa), all at 1 mg/ml. To analyze ER-DNA complexes by gel filtration, 35 μM of purified ER in 50 mM 150 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl2, 0.05% Tween 20, pH 7.5, were incubated with 20 ng of 32P-labeled oligonucleotide in presence or absence of [1H]estradiol (10-8 M) at room temperature for 30 min. The sample was then applied to the Superdex 200 column and eluted in the buffer described above (flow rate 0.25 ml/min). Radioactivity in fractions (0.25 ml) from the column was detected by liquid scintillation spectrometry.

RESULTS

Purification of hER—Commercially available, partially purified recombinant baculovirus-infected SF9 cell hER was used for this study. We further purified this protein to homogeneity using gel filtration and chromatography on a Ni2+-affinity column. As described previously (31), metal-affinity chromatography can be applied successfully for hER purification, hER binds to a Ni2+-resin without additional poly-His fusion. The affinity of this interaction is relatively high, and receptor can be eluted from the column with 100 mM imidazole. Purified ER is detected as one band on a Coomassie-stained SDS gel (data not shown). The apparent molecular mass of the purified receptor is 67 kDa. Western blot analyses demonstrates that this protein can be recognized by an anti-hER antibody (data not shown).

Purified hER Specifically Interacts with DNA—In vitro, purifed receptor binds with high affinity to a palindromic inverted repeat element containing the sequence AGGTCTTTTGTACGTCATCAGCTGACCTGAACTTACCCCCGAGCAA-3′, which is a specific binding site as follows: A1 + A2 + B = A1B + A2B (28).
directly repeated AGGTCA elements spaced by 3 bp (DR) which represents a high affinity binding site for the vitamin D3 receptor (35). The other oligonucleotide, derived from the mouse complement component C3 gene promoter (C3), which is regulated by estradiol (29). It contains a perfect AGGTCA and one element, AGTCTA different from consensus, positioned as an inverted repeat with a spacing of 3 bp. It has been demonstrated that minimal activity is associated with a single copy of the C3 enhancer element when this oligonucleotide is inserted 5' to a thymidine kinase promoter and cotransfected with human ER in to Ishikawa cells (36). Our results (Fig. 1) demonstrate that complexes of the same size as detected with Vit.A2 can be formed by the interaction between the hER and DR or C3 oligonucleotides in gel retardation assays. Complex quantitation using PhosphorImager demonstrated that the amount of shifted DR or C3 is more than 100 times lower than that for Vit.A2. This result implies that the affinity of the ER interaction with these oligonucleotides is significantly reduced compared with the Vit.A2.

**Analysis of ER-ERE Complexes by Gel Filtration**—To clarify the nature of the various ER-DNA complexes, we have examined them using analytical gel filtration chromatography. This technique is very useful for the evaluation of the apparent molecular weight and stoichiometry of different protein-DNA complexes, and this approach has been applied previously to the study of vitamin D3 receptor interaction with specific DNA (41).

Purified hER elutes from this column as a major peak with apparent mass of 140 kDa and several minor peaks with apparent mass of 70, 300, and 540 kDa (data not shown). This result suggests that the major molecular form of hER in solution is a dimer. The fact that other complexes besides monomers and dimers were found implies that hER has a tendency to aggregate. hER bound to [3H]estradiol elutes as two complexes, with apparent mass of 70 and 140 kDa (data not shown) with a dimer being the major molecular form of ER under these conditions.

To analyze ER-DNA complexes by gel filtration, the receptor was incubated with [3H]estradiol and then mixed with 32P-labeled oligonucleotide before this mixture was applied on Superdex 200 column. Radioactivity in fractions from the column was detected by liquid scintillation spectrometry.
(HEGO) in the presence of E₂ or 4(OH)-tamoxifen, preincubation at 4 or at 37 °C had little effect on DNA binding, whereas in the absence of ligand, or in the presence of ICI-182,780, incubation at 37 °C resulted in greatly reduced DNA binding. To further examine if hER demonstrates a ligand dependence for ER-ERE complex formation detectable by GSA, the receptor was incubated at 25 or at 37 °C in the absence or presence of E₂, 4-hydroxytamoxifen, raloxifene, or ICI-182,780, and then labeled DNA was added and incubation was continued at the same temperature. Complexes formed were analyzed by GSA (data not shown). Our results demonstrate that E₂ binding had little or no effect on the amount of ER-ERE complex formed at 25 °C, as detected by GSA. A similar result was obtained with 4-hydroxytamoxifen, raloxifene, and ICI-182,780. If, however, preincubation was performed at 37 °C, significant reduction of ER-ERE complex formation was observed in the absence of ligand or in presence of ICI-182,780. Therefore, the purified hER exhibits the same ligand dependence for DNA binding as wild type hER (HEGO) (38).

Analysis of ER-ERE Interaction by SPR Detector, BLAcore—Absence of detectable differences in the amount of formed ER-ERE complex by liganded or unliganded ER at 25 °C may also reflect the inability of GSA to detect differences in affinity of ligand-induced ER-ERE interaction. On the other hand, it
may be due to the fact that ligand binding affects the kinetics of ER-ERE interaction while having a minimal effect on affinity. To address these possibilities we have used real time analysis with BIAcore to study ligand effects on the kinetics of ER-ERE complex formation at 25 °C.

Experiments with the BIAcore usually start with the equilibration of the surface with running buffer (Fig. 3). There is an "injection jump" at the beginning and end of each injection due to the difference in the refractive index between the running and sample buffers (presumably due to small changes in salt concentration). Protein is injected during the "wash-in" phase. During the "wash-out" phase, the running buffer is injected across the flow cells. After approximately 15 s from the start or the end of injection, the data generated can be used for the kinetic analysis.

We approached our study by immobilization of 1094 RU of DR-oligonucleotide in FC 1, 943 RU of Vit.A 2 oligonucleotides in FC 2, and 1063 RU of C3 oligonucleotides in FC 3 on the surface of a sensor chip (see "Experimental Procedures"). Human ER bound with E2 was injected over the surfaces coated with these different oligonucleotides (Fig. 3). High affinity interaction was detected in the flow cell with Vit.A 2 immobilized. This is indicated by a fast increase of the refractive index, detectable after the injection jump in the beginning of injection (see Fig. 3, Vit.A 2). At the end of injection, a significant amount of ER is still bound to the surface, which can be detected as a difference between the refractive index of the injection over the surface with immobilized Vit.A 2 and the base line. At the same time binding of hER to immobilized streptavidin (data not shown), DR, or C3 surfaces was very low. Thus, the mass increase on the surface of the sensor chip in response to the injection of hER is due to specific receptor binding to DNA. Injection of ER over the surface with no immobilized DNA results in no detectable binding (data not shown).

To study how different ligands affect ER-ERE interactions, a surface with a gradient of immobilized Vit.A 2 was created. First a gradient of immobilized streptavidin in different flow cells was obtained by varying the streptavidin injection time as illustrated in Fig. 4A. As a result, surface with 2918, 1922, 1363, and 908 RU of streptavidin in FCs 1–4, respectively, was

**Fig. 5. Titration of the immobilized DNA with hER at different concentrations.** Serial injections of hER preincubated overnight with 10^{-6} M of E2 at protein concentrations of 35, 87.5, 175, and 270 nM were run over the 605 (A), 917 (B), 1290 (C), and 1817 RU (D) of Vit.A 2 immobilized on a surface of sensor chip.
Fig. 6. Estrogen receptor ligands modulate interaction with DNA. Overlaid sensograms of hER injections over the surface with 943 RU of immobilized Vit.A2. Unliganded hER and hER preincubated overnight with 10⁻⁶ M of E₂, 4(OH)-tamoxifen, raloxifene, and ICI-182,780 at protein concentration of 89.5 nM were used for this experiment.

Table I. Effect of the different compounds on kinetics of ERE-ER interaction

| Compound   | $k_d$     | $k_a$     | $k_{off}$ |
|------------|-----------|-----------|-----------|
| No ligand  | $5.06 \pm 1.3 \times 10^{-5}$ | $8.17 \pm 1.2 \times 10^{-4}$ | $6.19 \times 10^{-5}$ |
| 17α-Estradiol | $1.86 \pm 0.6 \times 10^{-3}$ | $9.62 \pm 0.4 \times 10^{-4}$ | $1.93 \times 10^{-5}$ |
| 17α-Ethynyl estradiol | $1.94 \pm 0.3 \times 10^{-5}$ | $1.05 \pm 0.4 \times 10^{-5}$ | $1.84 \times 10^{-5}$ |
| 4(OH)-Tamoxifen | $1.96 \pm 0.2 \times 10^{-3}$ | $8.78 \pm 0.5 \times 10^{-4}$ | $2.23 \times 10^{-5}$ |
| 3(OH)-Tamoxifen | $1.05 \pm 0.2 \times 10^{-3}$ | $7.50 \pm 0.3 \times 10^{-4}$ | $1.40 \times 10^{-5}$ |

Fig. 7. Chemical modifications of the ligand molecules affect receptor-DNA interactions. hER at 89.5 nM was preincubated overnight with 10⁻⁶ M of the 4(OH)-tamoxifen, 3(OH)-tamoxifen, raloxifene, zuclomiphen, and toremifene and injected (50 μl) over the surface with 943 RU of immobilized Vit.A2.

Table I obtained. Injection of 50 μl of biotinylated Vit.A2 at 33 ng/μl resulted in a surface with 1817, 1290, 917, and 605 RU of immobilized Vit.A2 in FCs 1–4, respectively (Fig. 4B).

Serial injections of unliganded ER and ER incubated overnight with 10⁻⁶ M of E₂, 4(OH)-tamoxifen, raloxifene, and ICI-182,780 at protein concentrations ranging from 35 to 270 nM were run over the sensor chip with immobilized gradient of the Vit.A2. Fig. 5 demonstrates overlaid sensograms of the injections of the hER at different protein concentrations, liganded with E₂, over the gradient of immobilized Vit.A2. Clear correlation between the change of refractive index and the level of immobilized DNA was obtained. Saturation of immobilized ERE with injected hER was reached for the hER liganded with 4(OH)-tamoxifen and E₂ at 270 nM hER. We found that there is a linear relationship between the surface density (amount of immobilized DNA) and the saturation response (refractive index at the end of injection) obtained with 270 nM injection of hER in the interval of the DNA densities used for this experiment (data not shown). Calculated stoichiometry for the formed ER-ERE complex (42) was 1:1.92 ± 0.11. This result confirms that hER binds specific oligonucleotide-Vit.A2 as a homodimer.

Fig. 6 shows overlaid sensograms obtained by injections of 50 μl of unliganded hER and hER liganded with E₂, 4(OH)-tamoxifien, raloxifene, and ICI-182,780 at receptor concentration of 89.5 nM over the FC with 900 RU of VitA2 immobilized. It can be seen that different ligands significantly affect the ER-ERE interaction. Relative to unliganded or ER liganded with ICI-182,780, much more of the ER-ERE complex is formed when ER is liganded with E₂ or 4(OH)-tamoxifen. It can also be seen that this complex is less stable than the complex induced by ICI-182,780 and raloxifene.

Sensograms of the injections of ligand and unliganded hER over the gradient surface with immobilized Vit.A2 were analyzed using the BIAEvaluation 2.1 program, as described (28). Values of apparent dissociation, association, and affinity rate constants determined for ER-ERE interaction in the absence and presence of different ligands are summarized in Table I. It is interesting that E₂ binding (pure agonist) is inducing fast formation ($k_d = 9.62 \times 10^4$ M⁻¹ s⁻¹) of unstable ER-ERE complex ($k_d = 1.86 \times 10^3$ s⁻¹), at the same time ICI-182,780 binding (pure antagonist) is inducing slow formation ($k_d = 1.48 \times 10^3$ M⁻¹ s⁻¹) of a very stable ER-ERE complex ($k_d = 6.35 \times 10^4$ s⁻¹).

We were also interested in examining if derivatives of the same compound may have a different effect on ER-ERE interactions. hER was preincubated with the analogs of 4(OH)-tamoxifen at 10⁻⁶ M (tamoxifen, 3(OH)-tamoxifen, 4(OH)-tamoxifen, zuclomiphen, and toremifene) overnight and then used for serial injections at different protein concentrations over the surface of a sensor chip carrying immobilized Vit.A2. The effect of these compounds on the kinetics of hER interaction with the ERE was examined as previously described (28). Values of the apparent association and dissociation rates are summarized in Table I. Fig. 7 presents overlaid sensograms of hER liganded with the analogs of 4(OH)-tamoxifen injected over a surface with ER immobilized. It is clear that even a small chemical modification of the ligand (position of OH group in the molecule of tamoxifen), presumably modulating receptor’s conformation, may affect receptor-DNA interactions. It can be seen that stability of the ER-ERE complex induced by the 4(OH)-tamoxifen is much lower (see Table I) than for the complexes induced by 3(OH)-tamoxifen and tamoxifen. It is interesting that on consensus ERE, 4(OH)-tamoxifen is a more potent agonist than other analogs of tamoxifen used in this study.
DISCUSSION

We have used the SPR technology to study the DNA binding by the hER, in the absence and presence of estradiol, analogs of tamoxifen, raloxifene, and ICI-182,780. This approach allows detection of the receptor-DNA complex formation in real time. This information can then be fit into a mathematical model describing this interaction to obtain kinetic and thermodynamic rates of the receptor-DNA association and dissociation. Therefore, it was important to use pure hER and to know the nature of the complexes that could be formed by ER-ERE interaction.

Commercially available, partially purified recombinant baculovirus-infected Sf9 cells hER were used for this study. We further purified this protein to homogeneity. Purified hER specifically binds to the Xenopus vitellogenin A2 gene palindromic ERE in vitro and can be recognized by a specific anti-hER antibody. Analytical gel filtration analysis showed that the predominant molecular form of this hER preparation, in the presence or absence of estrogen, at protein concentrations ranging from 40 to 300 nM is a homodimer. Monomeric hER and complexes with greater than 2 orders of oligomerization were also identified, and this result is in agreement with previous studies (38, 43, 44). In the presence of specific labeled oligonucleotide, derived from the vitellogenin A2 gene of X. laevis response promoter, homodimer (hER)ERE complex was detected. Some of the labeled DNA, approximately 3–5%, was eluted close to a column void volume, which suggests that oligomeric hER-ERE complexes could also be formed.

Gel shift analysis of hER interaction with DNA clearly demonstrated ligand dependence at 37 °C. When incubated at 25 °C, there were no differences found between amounts of ER-ERE complex formed in the presence and absence of different ligands as detected by gel shift analysis. These observations are in keeping with a previous study of Metzger et al. (38) who reported that wild type hER when incubated at 25 °C binds DNA irrespective of the presence or absence of estrogen and that in absence of ligand, or in the presence of ICI-182,780, incubation at 37 °C resulted in greatly reduced DNA binding.

We have hypothesized that ligand binding may affect the kinetics of ER interaction with DNA while having a minimal effect on its affinity. It was previously reported that binding of hormone accelerates the kinetics of glucocorticoid and progesterone receptor binding to DNA (33) and that binding of 1,25-(OH)2D3 affects the dimerization status and kinetic of DNA binding by the vitamin D3 receptor (28).

To address our hypothesis, we have used the surface plasmon resonance detector BLACore to measure the effect of various ligands on the hER interaction with DNA. Previous studies (28, 45–48) have demonstrated that surface plasmon resonance-based methodology can be effectively used to study protein-DNA interactions. We found that this approach gives adequate information about hER interaction with DNA. High affinity binding was found to the Xenopus vitellogenin A2 gene palindromic ERE. Low levels of interaction were detected using oligonucleotides designed as two directly repeated AGGTCA elements spaced by 3 bp (DR) and an oligonucleotide that contains a perfect AGGTCA and one element AGTCTA different from consensus, positioned as an inverted repeat with a spacing of 3 bp (C3). Similar results were obtained using gel shift analysis.

To obtain kinetic rates of ER-ERE interaction, gradient surfaces of immobilized DNA were used. We show that immobilized DNA can be saturated with hER which implies that the interaction studied is specific. Based on the signal at equilibrium we calculated the stoichiometry between ER and ERE. This result reconfirms that ER binds DNA as a homodimer.

Using real time interaction analysis, we found that ligand binding dramatically affects kinetics of hER interaction with the VitA2 response element. We also found that binding of estradiol induces rapid formation of an unstable ER-ERE complex and, furthermore, binding of “pure” antagonist such as ICI-182,780 results in a slow formation (kd is approximately 100 times lower) and a very stable receptor-DNA complex (kd is almost 100 times lower). Most importantly, we demonstrate that there is a good correlation between the kinetics of hER-ERE interaction induced by a hormone and its biological effect. For example, the stability of ER-VitA2 complex, which can be characterized by its kd, can be decreased from E2 (pure agonist) to 17α-ethynyl estradiol > 4(3H)-tamoxifen (partial agonist) > raloxifene (partial antagonist) > ICI-182,780 (pure antagonist). It is interesting that this order corresponds to the increase in the antagonistic activity of these compounds on consensus ERE (49).²

Currently, we do not know the precise mechanism whereby differences in kinetics of receptor-DNA interaction induced by the binding of the ligand could be related to the observed behavior of the estrogen receptor in vivo. It is clear, however, that binding of estradiol accelerates the ER turnover more than 50-fold, compared with unliganded ER, and more than 1000-fold compared with ER liganded with ICI-182,780. Therefore, ligand binding inducing conformational changes is not just modulating receptor’s interaction with other transcriptional factors (11–13). Our data suggest that these changes are affecting the kinetics of receptor-DNA binding which regulates the frequency of receptor-DNA complex formation. We hypothesize that a correlation exists between the rate of gene transcription and the frequency of receptor-DNA complex formation.

Various manifestations of the ligand binding including dissociation from hsp90 and p59, modulation of receptor dimerization status, modification of the kinetics of receptor-DNA interaction, and effects on receptor interaction with transcriptional intermediary factors represent different levels at which ligands of steroid receptors may affect transcriptional regulation. We have investigated the role of the estrogen receptor ligands in receptor’s interaction with the consensus ERE. The pattern that we found may, however, be different for other DNA elements or it may be modified by other transcription factors interacting with estrogen receptors, which probably can explain the tissue-specific effect of different ligands of estrogen receptor.

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