Characterization of DNA-binding of the Rat Uterine Estrogen Receptor

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ABSTRACT. We characterized DNA-binding of the estrogen receptor from rat uterus by UV cross-linking and gel retardation analysis. The nuclear estriol- and LY139478 - receptor complexes from rat uterus were bound as a dimer as well as the other liganded nuclear receptors with a synthetic oligonucleotide containing a *Xenopus* vitellogenin A2 estrogen response element (VRE) which was substituted with 5-bromo-2'-deoxyuridine by UV irradiation. In addition, the nuclear antiestrogen-receptor complexes showed much higher binding ability with VRE than the nuclear estrogen-receptor complex under a high salt condition by gel retardation analysis.

Key words : estrogen receptor — DNA-binding — UV cross-linking — gel retardation analysis — antiestrogen

Estrogen acts as a transcription regulator by mediation of a nuclear receptor in target cells. The estrogen-receptor complex specifically interacts with a *cis*-acting estrogen response element (ERE) usually in the 5' up-stream region of the target gene.[1)]

The action mechanisms of not only estrogens, such as 17β-estradiol (E_2) and estriol (E_3) (a weak estrogen), but also estrogen antagonists (antiestrogens), such as 4-hydroxytamoxifen (OHT) (a potent tamoxifen metabolite), ICI164384 (ICI) (a pure antagonist) and LY139478 (HCl salt of LY117018) (LY), are not yet clearly understood.[2)] Physicochemical differences in the estrogen receptor binding with estrogens and antiestrogens have been reported. The nuclear estradiol-receptor complex has a lower sedimentation rate on ultracentrifugation analysis than the OHT- or ICI-receptor complexes. The antiestrogen-receptor complex appears to run 10% more slowly than the estrogen-receptor complex on gel retardation analysis. Tamoxifen inhibits TAF-2 activity of the C-terminal E region (the hormone-binding domain) of the receptor. This is one of two independent transcriptional activation functions.[3)] ICI was reported to induce rapid loss of the receptor protein in the mouse uterus.[4)] In eliciting more details regarding the action mechanisms of estrogens and antiestrogens through elucidation of the DNA-binding state of the receptors, Ikeda and Kusaka[5)] reported that the nuclear E_2-, ICI- and OHT-receptor complexes and the soluble

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receptor from rat uterus (rE_2ERn, rICIERn, rOHTERn and rMoERs, respectively) were cross-linked with 5-bromo-2'-deoxyuridine substituted ERE as the receptor dimer. On the electrophoretic SDS-gels the rMoERs was observed as a slower mobile complex than the simple receptor dimer. In addition, unknown bands (Mr = 50-55 kDa, 39-41 kDa) were concomitantly observed with those of the nuclear receptors.

In this paper, the DNA binding of the nuclear estrogen- and antiestrogen-receptor complexes from rat uterus was examined by UV cross-linking and gel retardation analysis.

EXPERIMENTAL

Materials

[α-^{33}P]dATP (> 2000 Ci/mmol) was obtained from NEN Research Products (Boston, MA, USA). 5-Bromo-2'-deoxyuridine-5'-triphosphate(BrdU) and estriol (E_3) were purchased from Sigma Co. (St. Louis, MO, USA). LY139478 was a gift from Eli Lilly Co. (Indianapolis, IN, USA). A color marker [rabbit muscle myosin (205 kDa), E. coli β-galactosidase (116 kDa), bovine serum albumin (66 KDa), chicken egg ovalbumin (45 kDa) and carbonic anhydrase (29 kDa)] (Sigma Co.) were used as standards in the Laemmli SDS-PAGE system. The monoclonal anti-estrogen receptor α antibody H222 was from Abbott Laboratories (Chicago, IL, USA).[6,7]

Buffer

The following buffer was used[5,8]: TEGM buffer (10 mM Tris-HCl, 1 mM EDTA, 10% (v/v) glycerol, 3 mM $MgCl_2$, pH 7.4). The buffer contained 3 mM EGTA, 50 μg/ml each of leupeptin (Boehringer Mannheim Co., Mannheim, Germany), antipain (Sigma Co.), chymostatin (Sigma Co.), and soybean trypsin inhibitor (Boehringer Mannheim Co.) was designated as TEGMPI.

Animal and tissue preparation

Female Wistar rats (20-21 days old) were obtained from Charles River Co. (Yokohama, Japan). The experiments were conducted according to the "Guide for the Care and Use of Laboratory Animals" of Kawasaki Medical School. After the animals had been treated for 20 min with 20 μg/kg E_3 or for 30 min with 150 μg/kg LY or with vehicle alone, they were sacrificed by cervical dislocation. The uteri were removed and immediately frozen in liquid nitrogen. They were used immediately after removal or stored at -70°C until use.

Preparation of the nuclear receptors

All operations were carried out at 0-4°C unless stated otherwise. rE_2ERn, rICIERn and rOHTERn were prepared as described previously.[5,6] The uteri from the rats treated with E_3 or LY were homogenized in the TEGMPI buffer. These nuclear receptors (designated as rE_3ERn and rLYERn, respectively) were also prepared as described previouly.[5,8]

Protein concentrations were determined by the Bio-Rad protein assay based on the Bradford method using rabbit γ-globulin as the protein standard.[9] The assay of total estradiol receptor was performed according to the method of Sica

et al.[10] Values were the means of duplicate determinations.

Synthetic oligonucleotides

A wild type 25-base pair synthetic oligonucleotide containing a *Xenopus* vitellogenin A2 ERE (VRE), [^{33}P]VRE and [^{33}P]5-bromo-2'-deoxyuridine substituted VRE (BrdUVRE) were prepared as descibed previously.[5] Briefly, the synthetic oligonucleotide 5' TCAGGTCACTGTGACCTGACTTTGG 3' was annealed to the complementary primer 5' CCAAAGTC 3'. The annealed oligonucleotides were end-filled with klenow fragment.

Gel retardation analysis

Specific binding of the receptor to [^{33}P]VRE and [^{33}P]BrdUVRE was performed as described previously.[5,8,11] rE_2ERn (6.8 fmol receptor, 49 μg protein), rE_3ERn (7 fmol receptor, 32 μg protein), rOHTERn (4.9 fmol receptor, 23 μg protein), rICIERn (2 fmol receptor, 23 μg protein) and rLYRn (3.8 fmol receptor, 23 μg protein) were incubated with [^{33}P]VRE or [^{33}P] BrdUVRE (50,000-300,000 dpm, 1.11 pM). Autoradiography was done with a Bio-imaging Analyzer BAS 2000 (Fuji Film Co., Tokyo, Japan). The amount of the receptor bound [^{33}P]VRE was measured by the density (PSL value) of the specific band on autoradiograms using the quantify mode of the Bio-imaging Analyzer. On this experimental scale, the radioactivity of [^{33}P] DNA was in proportion to the PSL value of the band. The backgrounds were subtracted from the measured values.

UV cross-linking

UV cross-linking of the receptors to [^{33}P]BrdUVRE was performed as bescribed previously.[5] Briefly, the complexes of [^{33}P]BrdUVRE and the receptors were irradiated with a UV lamp on the retardation gels on ice. The specific retarded complexes were electrophoresed in SDS-PAGE and autoradiographed.

RESULTS AND DISCUSSION

UV cross-linking of rE_3ERn and rLYERn

Specific binding of rE_3ERn and rLYERn to [^{33}P]BrdUVRE was determined by using gel retardation analysis. The retarded bands for rE_3ERn and rLYERn were observed. The bands disappeared as a result of competition with excess unlabeled VRE. A super-shift of the retarded bands was observed by incubation with H222 (data not shown). These showed that the retarded band contained the receptor α-BrdUVRE complex. After UV-irradiation, the bands were excised and analyzed by SDS-PAGE. In rE_3ERn, a band of Mr = 140-150 kDa was observed as a major band on SDS-gel (Fig 1 A). The molecular weights of the rat estrogen receptor α were calculated to be 67029 daltons.[12] Therefore, this is thought to correspond to an estrogen receptor α dimer. This finding suggests that estriol acts through binding to DNA as the receptor α dimer. In rLYERn, bands of Mr = 170, 120, 44 and 33 kDa were observed on SDS-gel (Fig 1 B). The band of Mr = 120 kDa (130 kDa in the other set of the same experiment) corresponds to the receptor α dimer as mentioned above. The unknown bands of Mr = 50 to 55 kDa, 39 to 41 kDa

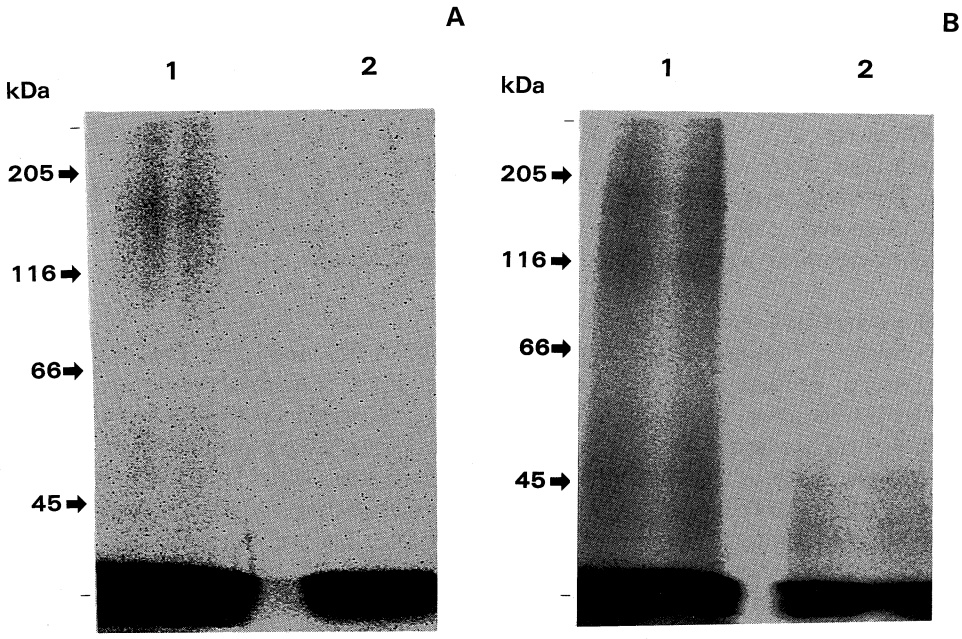

Fig 1. UV cross-linking of rE_3ERn and rLYERn.
rE_3ERn (A) and rLYERn (B) was incubated with [^{33}P]BrdUVRE in the absence (lane 1) or presence (lane 2) of excess unlabeled VRE and electrophoresed through non-denatured gel (5.6%) under the gel retardation condition. The receptor-[^{33}P]BrdUVRE complexes were irradiated *in situ* on the gel for 30 min. Specific bands were excised from the gels. The extracts were analyzed by SDS-8% PAGE and autoradiographed. The position of molecular mass markers is indicated on the left.

on the electrophoretic SDS-gel were observed with those of the rE_2ERn, rICIERn and rOHTERn.[5] However, such bands were not observed in the overexpressed recombinant estrogen receptor α in COS-7 cells.[13] The bands of Mr = 44 and 33 kDa in rLYERn correspond to those bands. In rE_3ERn, those bands were observed slightly. Therefore, the bands were thought to be from target cell specific factors. The band of Mr = 170 kDa was not observed in the nuclear receptor complexes with the other ligands such as E_2, OHT, ICI, LY and E_3. This may be indicative of the difference in DNA binding with rLYERn from the other ligand-receptor complexes. All liganded receptors we examined were found to bind as receptor α dimers to BrdUERE *in vitro*. This result suggests that estrogen- and antiestrogen-receptor complexes act by binding to ERE as dimers in the cells.

Effects of KCl concentration on receptor binding to DNA

The effects of KCl concentration on the VRE binding of rE_2ERn, rE_3ERn, rICIERn, rLYERn and rOHTERn were determined by gel retardation analysis (Figs 2 A-E). Specific binding of rE_2ERn was fairly decreased in the presence of more than 0.4 M KCl (Fig 2 A). Binding of the estradiol-receptor complex to ERE has been reported to have decreased in the presence of 0.2 to 0.5 M KCl.[14,15] Our observations are consistent with these reports. For rOHTERn, rICIERn and rLYERn (antiestrogens), the specific binding decreased in the

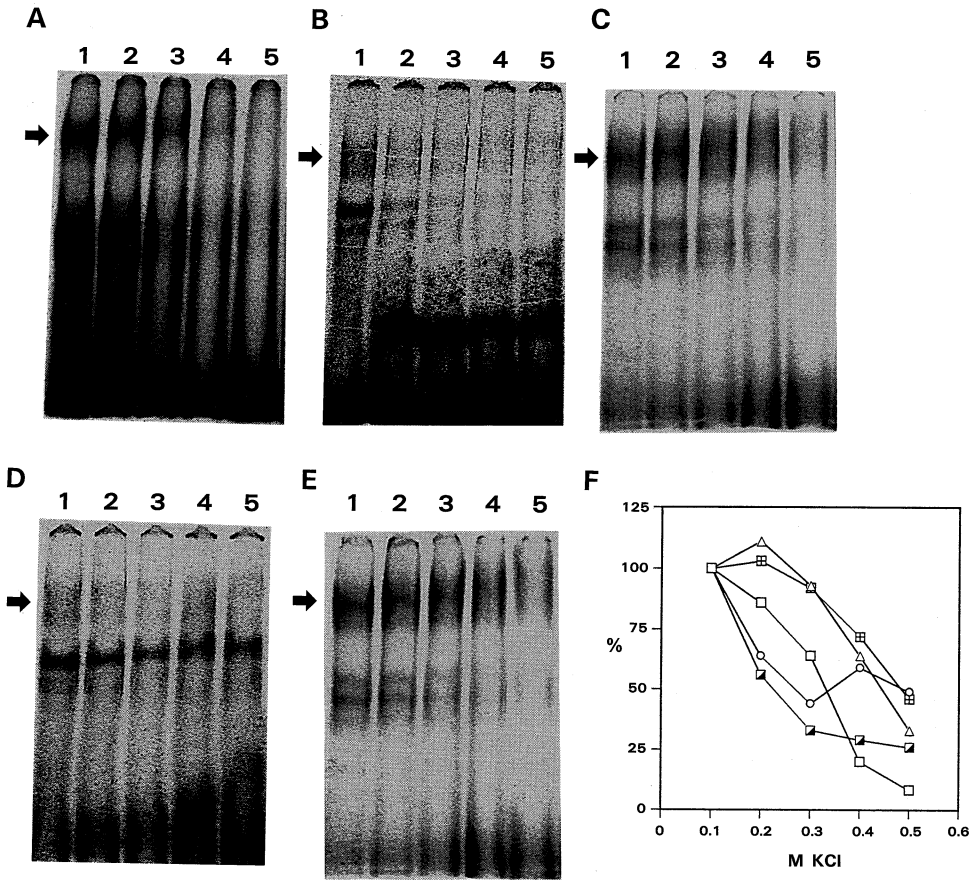

Fig 2. Effect of KCl concentration on rat uterine nuclear receptor binding to DNA. rE_2ERn (A), rE_3ERn (B), rOHTERn (C), rLYERn (D) and rICIERn (E) were incubated with [^{33}P]VRE in the presence of 0.1 (lane 1), 0.2 (lane 2), (lane 3), 0.4 (lane 4) and 0.5 (lane 5) M KCl, electrophoresed through non-denatured PAGE (5.6%) under the gel retardation condition and autoradiographed. The arrows indicate specific bands. The radioactivity of each specific band {rE_2ERn (□), rE_3ERn (◪), rICIERn (⊞), rLYERn (○), rOHTERn (△)} was expressed as the percentage of each PSL value of the band in the presence of 0.1 M KCl (F).

presence of 0.4 to 0.5 M KCl (Figs 2 C, D, E). But the inhibition in DNA-binding of the nuclear antiestrogen-receptor complexes in a high salt condition was not seen as much as of rE_2ERn. The radioactivity of each specific binding band was quantified and expressed as a percentage of each PSL value from the experiment achieved in the presence of 0.1 M KCl (Fig 2 F). Specific binding of rOHTERn and rICIERn were optimal in the presence of 0.2 M KCl. The profile of specific binding of rE_3ERn was more like that of rE_2 ERn than those of rOHTERn, rLYERn and rICIERn. The profile in rLYERn differed from those of the other liganded receptors. It was suggested that estrogens and antiestrogens induce different conformational changes in DNA-receptor complexes when they bound to the receptor.[16] In this study, ionic interaction with DNA was also implied the difference between the

estrogen- and antiestrogen-receptor complexes.

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