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IDENTIFICATION OF UNOCCUPIED BUT TRANSFORMED NUCLEAR ESTROGEN RECEPTOR IN CULTURED MOUSE LEYDIG CELL

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Summary—The molecular forms of estrogen receptor (ER) in estrogen-responsive mouse Leydig cell line (B-1) have been examined in relation to their biological activity. ER was predominantly recovered in the nuclear fraction upon homogenization even after cells were precultured in the absence of E_2 and Phenol Red. This unoccupied nuclear ER (ERn) whose hormone binding ability was extremely thermostable could be extracted with 0.4 M KCl. This stability enabled us to determine hydrodynamic parameters in the ligand-free condition. The Stokes radius and sedimentation constant of this ERn in high salt condition were 5.5 nm and 6.0S, respectively, resulting in its molecular weight of 140,000. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of ER labeled with [^{3}H]tamoxifen aziridine gave a single band of 65,000 Da, indicating that this ERn had a oligomer structure similar to that of transformed nuclear ER complexed with estrogen in the putative target cells. Therefore, we further examined the possibility that this ERn in B-1 cells can activate estrogen-responsive genes without any aid from estrogen. Estrogen responsive element-thymidine kinase promoter-chloramphenicol acetyltransferase fusion gene (ERE-tk-CAT) was transfected into B-1 cells. CAT activity was enhanced only in cells stimulated with estrogen. It may be concluded from these results that transformed ERn can be formed in the absence of estrogen but that binding to estrogen may be required in order to exert its biological activity.

INTRODUCTION

According to the classical two-step mechanism, steroid hormone binds to the cytoplasmic receptor and this complex migrates into the nucleus where it interacts with the chromatin to modulate gene expression [1]. Subsequent to binding of the hormone to receptors, the receptor protein is believed to undergo some kind of conformational change (defined as transformation in this manuscript) before translocation to the nucleus. In the case of estrogen receptor (ER), transformation which occurs in a temperature- and ligand-dependent manner is accompanied by an alteration in its molecular weight from 70,000 to 140,000 when analyzed in a high salt condition [2, 3]. Most evidence on this two-step mechanism is derived from biochemical studies. Recent immunohistochemical studies reveals that ER may be localized in the nucleus even in the absence of ligand [4], although autoradiographic studies with [^{3}H]estradiol (E_2) in rat uterus have shown temperature-dependent binding of E_2 to nucleus [5].

Different molecular forms of ER have been reported under various *in vitro* conditions. However, the precise physicochemical nature of the "native" receptor is still unknown. What are the subcellular sites for biologically active and inactive forms of ER? Recently, evidence showing that steroid receptors can stimulate gene expression as a homodimer have been presented [6, 7]. Transforemd ER with a molecular weight of ~140 K (classically termed as a "5S" form [2]) is also believed to be a homodimer [8, 9]. These observations would suggest that this transformation process is crucially important for eliciting the estrogen action. In spite of extensive studies, this transformation mechanism is yet to be ascertained. Although ligand binding is considered to be essential for this transformation, recent studies have revealed that "nuclear translocation" of ER in MCF-7 cells is enhanced under an intact cell condition by estrogen-unrelated compounds such as dithiothreitol or dibutyryl cGMP [10], raising the interesting possibility that ER transformation may occur without any aid of estrogen in a certain condition or in some target cells. Identification of such a unique ER form, if present, would provide us with the excellent opportunity to further discuss the relationship between ER forms and their biological activities. During studies on the estrogen action mechanism in transformed murine Leydig cell line (B-1), unoccupied nuclear ER with a oligomer structure was detected. In addition,

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its biological activity was examined using B-1 cells transfected with a reporter plasmid.

EXPERIMENTAL

Chemicals

17β-[2,4,6,7-^{3}H]estradiol (E_2) (SA 95 Ci/mmol) and ^{14}C-labeled protein markers for gel electrophoresis were obtained from New England Nuclear (Boston, Mass). [ring-^{3}H]tamoxifen aziridine (TA) (SA 26 Ci/mmol) and [^{14}C]chloramphenicol (SA 60 mCi/mmol, CAT assay grade) were from Amersham International plc (Buckinghamshire, England). The following biochemicals were used; diethylstilbestrol (DES), E_2, bovine serum albumin (BSA) (crystalized and lyophilized), bovine insulin, protease-free DNase I (Type I) (Sigma Chemical Co., St Louis, Mo.), leupeptin (Peptide Institute, Osaka, Japan); Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden) and calibration proteins from gel filtration chromatography (Boehringer Mannheim, West Germany). Hydroxyappatite (HAP) and materials for gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, Calif.). Enzymeimmunoassay (EIA) kit for ER and 4-hydroxytamoxifen (OHT) were kindly supplied by Dainabot Inc. (Tokyo, Japan) and ICI Pharma (Osaka, Japan), respectively. The restriction enzymes were from Toyobo Ltd (Osaka, Japan). The other chemicals used here were of analytical grade.

Cell culture

The cultured cell line (B-1) was established as described previously [11]. Cells were routinely maintained in Eagle minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 10^{-8} M E_2 and 0.1 μg/ml insulin (Medium-I). To prepare the estrogen-free cells for ER analysis, cells were precultured in Phenol Red-free MEM supplemented with 0.1 μg/ml insulin and 10% FBS pretreated with dextran (0.01%; w/v)-coated charcoal (1%; w/v) (DCC) (Medium-II) [12].

Subcellular fractionation

All procedures were carried out at 0–4°C unless specified otherwise. The pellet of the cultured cells was homogenized in 10^8 cells/ml of Buffer-I (0.32 M sucrose, 10 mM Tris, 10 mM Na_2MoO_4, 10 mM dithiothreitol (DTT), 0.5 mM leupeptin, pH 7.4 at 20°C). The homogenate was centrifuged at 800 *g* for 5 min. The supernatant was recentrifuged at 104,000 *g* for 60 min to obtain the cytosol. To prepare the isolated nuclei, the pellets from 800 *g* centrifugation were washed with Buffer-I and then resuspended in 0.1% (w/w) Triton X-100 in Buffer-I. After standing for 10 min, the nuclei were further purified by centrifugation at 15,000 *g* through discontinuous sucrose density centrifugation as reported previously [13]. The recovery of nuclei was always corrected by measurement of DNA. Both methods gave the identical results in terms of subcellular localization of ER (data not shown). In view of the fact that the higher DNA recovery (approximately 60%) was achieved by the low speed centrifugation method in comparison with that (30%) of the discontinuous sucrose density gradient method, the low speed centrifugation method was mainly used. The isolated nuclei were resuspended in the original volume of TEMMo-K buffer (0.4 M KCl, 10 mM Tris, 1.5 mM EDTA, 2 mM mercaptoethanol, 10 mM Na_2MoO_4, 10 mM DTT, 0.5 mM leupeptin, pH 7.4 at 20°C). The nuclear suspension in TEMMo-K buffer was kept at 4°C for 60 min and then centrifuged at 15,000 *g* for 10 min. The supernatant was used as the nuclear extract. The isolated nuclei were also suspended in Buffer-I containing 500 μg/ml DNaseI and 5 mM $MgCl_2$ at 4°C for 1 h to solubilize ER. The supernatant of 10,000 *g* centrifugation for 10 min was referred to as the DNaseI solubilized fraction.

ER determination

To determine the ER content, cytosol, nuclear extract and isolated nuclei were incubated with 5 nM [^{3}H]$E_2 \pm 1$ μM DES for 18 h at 4°C. Then, 0.2 ml of HAP slurry was added to separate [^{3}H]E_2-ER from free [^{3}H]E_2. After being washed three times with Buffer-I, associated radioactivity was measured as described previously [14]. In other experiments, nuclear extract, cytosol or DNase I-solubilized fraction was mixed with 0.2 ml of HAP slurry (50%, v/v). After standing at 4°C for 15 min with intermittent shaking, HAP was centrifuged at 500 *g* for 3 min and washed with Buffer-I, followed by resuspending in the original volume of Buffer-I. This HAP-suspension was served to [^{3}H]E_2 binding assay. For thermostability experiments, HAP-immobilized receptors were incubated for 1–3 h at 30–45°C without ligand, and then incubated with 5 nM [^{3}H]$E_2 \pm 1$ μM DES for 18 h at 4°C. Enzymeimmunoassay (EIA) was also performed to characterize ER as published before [15].

Analysis of physicochemical parameters

The hydrodynamic parameters of ER were determined by gel filtration and sucrose density gradient analysis. The aliquots (2 ml) of samples were applied on a Sephacryl S-300 column (2.6 × 47 cm) preequibriated with TEMMo-K buffer. The flow rate was kept at 10 ml/h using the upward flow adaptor. The column was calibrated with the following standards of known Stokes radii; cytochrome c (1.8 nm), BSA (3.55 nm), aldolase (4.5 nm), ferritin (6.1 nm). The sedimentation constant of ER was analyzed using 5 to 20% (w/v) sucrose density gradients in TEMMo-K buffer. The fluorescent BSA was included in all gradients as an internal marker [14].

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

To covalently label ER with [^{3}H]TA, B-1 cells were incubated with 10 nM [^{3}H]TA ± 1 μM DES at 37°C for 60 min. After incubation, the nuclear extract was prepared as described above. The trichloroacetic acid-precipitable material was then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (9% acrylamide) using Laemmli system [16] and fluorography as published previously [17].

Reporter plasmid construction

The prasmid psV_2CAT was digested with BamHI and HindIII, and then BamHI/HindIII fragments were treated with T4 DNA polymerase to generate a blunt end. This CAT containing fragment was cloned to the HincII site of pUC18. The plasmid pMT-TK, which was kindly supplied from Dr R. D. Palmiter [18], was digested with PvuII and BglIII to obtain the herpes simplex thymidine kinase promoter (tk promoter). After PvuII/BglIII sites were "filled in" as described above, tk promoter was inserted to pUC18-CAT, which was digested with XbaI. Synthetic oligonucleotide which contains estrogen responsive element (ERE) [19]

5′GATCAGGTCACTGTGACCTA 3′
3′TCCAGTGACACTGGATCTAG 5′

was synthesized and ligated to the BamHI site upstream of pUC18-tk-CAT. Thus, reporter plasmid, ERE-tk-CAT was constructed.

Transfection and CAT assay

Subconfluent B-1 cells were collected. After being washed twice with PBS (272 mM sucrose, 7 mM phosphate buffer, 1 mM $MgCl_2$ pH 7.4), cells were suspended in PBS at a cell density of 1.25×10^7 cells/ml. Aliquots (0.8 ml) of the cell suspension were transferred into the Gene Pulser Cuvette (Bio-Rad) with 40 μg of plasmid DNA (ERE-tk-CAT) and 40 μg of sonicated calf thymus DNA. After standing on ice for 10 min, cells were twice exposed to a 300 V pulse. These cells were then placed on to culture dishes with or without hormone in Medium-II. After 48 h, cells were collected and suspended with 120 μl of 0.25 mM Tris–HCl, pH 7.8, and lysed by freezing–thawing. The supernatant of 10,000 *g* for 10 min was used as a cell extract. The extract was incubated with 0.1 μCi [^{14}C]chloramphenicol and 20 μl of 4 mM acetyl-CoA for 1 h at 37°C to determine CAT enzyme activity. The reaction products were separated by thin-layer (TLC) chromatography, and the TLC plate was autoradiographed [20].

RESULTS

Subcellular distribution of ER

Unoccupied ER in B-1 cells was measured in the cytosol and nuclear fraction. B-1 cells which had been cultured in Medium II (E_2- and Phenol Red-free) for 6 days were homogenized. [^{3}H]E_2 binding assay was performed at 4°C. High affinity E_2 binder was mainly recovered in the nuclear fraction (95% in the nuclear fraction). The presence of 30 μM Phenol Red did not significantly alter the subcellular distribution (Table 1). The K_d (equilibrium dissociation constant) values of these receptors in the cytosol and nuclear fractions were 2–4 nM and 0.2–0.5 nM ($n = 3$), respectively. The treatment of the crude nuclei with 0.4 M KCl resulted in solubilization of 30–50% of the nuclear receptor without change of the K_d.

Table 1. Subcellular distribution of E_2 binding components

	fmol/10^6 cell (A)	(B)
Cytosol	0.71 ± 0.06	0.21 ± 0.03
Nucleus	6.79 ± 0.71	6.29 ± 2.39
Nuclear extract	2.53 ± 0.17	2.40 ± 0.32

The subcellular fractionation was performed as described in Experimental. The aliquots of the samples were incubated with 5 nM [^{3}H]E_2 ± 1 μM radioinert DES at 4°C for 18 h in a final volume of 0.25 ml. After incubations, 0.2 ml of a HAP slurry was added to all tubes in order to measure the bound form of [^{3}H]E_2. (A) Culture in Medium-II. (B) culture in Medium-II plus 30 μM Phenol Red. The values (mean ± SE) were obtained by three separate experiments.

In order to ascertain that predominant nuclear ER (ERn) is not occupied by estrogen, E_2 binding was determined at low and elevated temperatures. The solubilized ER was immobilized on to HAP as described in Experimental. Specific [^{3}H]E_2 binding obtained by incubation at 4°C for 18 h was similar to that at 37°C for 2 h (Fig. 1A). When purified nuclei

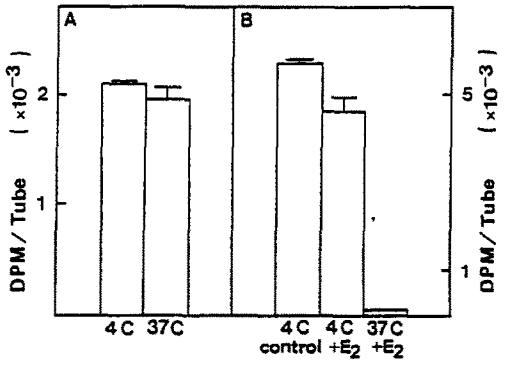

Fig. 1. Evidence of the presence of unoccupied ERn in B-1 cells. (A) Lack of temperature effects on [^{3}H]E_2 binding of ERn. Nuclear extract was prepared from cells cultured in Medium-II. ERn was immobilized on to HAP, and then suspended in Buffer-I. Specific [^{3}H]E_2 binding was determined by the incubation of HAP slurry (0.2 ml) with 5 nM [^{3}H]E_2 at 37°C for 2 h or at 4°C for 18 h. (B) Insignificant amount of ligand exchange in the nuclear extract at 4°C. Cells were treated with 5 nM [^{3}H]E_2 for 1 h. ERn was immobilized on to HAP. HAP suspension (0.4 ml) in Buffer-I was incubated at 4°C for 18 h without ligand as a control, or incubated with 5 nM unlabeled E_2 for 18 h at 4°C or for 1 h at 37°C. Radioactivity retained on HAP was measured after three times washing with Buffer-I. Results were expressed as mean ± SE of three experiments.

were measured for E_2 binding, [^{3}H]E_2 binding of 4°C was also similar to that at 37°C (data not shown). To eliminate the possibility that ligand exchange occurs even at 4°C, the following experiments were carried out. B-1 cells were first incubated with [^{3}H]E_2 at 37°C. Then, [^{3}H]E_2–ER complexes were extracted with 0.4 M KCl, and incubated with 10 nM radioinert E_2 at 4°C for 18 h. A large part of [^{3}H]E_2 associated with ER was not replaced by radioinert E_2. On the other hand, the incubation with radioinert E_2 at 37°C for 1 h resulted in a disappearance of the labeled receptor (Fig. 1B). These results indicated that ERn in B-1 cells was unoccupied.

Thermostability of ER in each fraction

In general, hormone binding ability of ER is unstable at elevated temperatures. We examined the thermal stability of ER by an exposure of various subcellular fractions to elevated temperatures in the absence of ligands. As shown in Fig. 2, KCl-extracted ERn was stable at 30–37°C, whereas approximately half of the cytosolic receptors lost their E_2 binding ability after incubation at 37°C. Thus, cytosolic and nuclear receptors showed different stability against thermal stress. Unoccupied ERn was also solubilized by DNaseI treatment. Treatment of the nuclear pellet with 500 g/ml DNaseI can solubilize 20% of ERn. However, this ERn solubilized by DNaseI was unstable at 30–37°C.

Physicochemical parameters of nuclear ER

This stability of KCl-extracted ER enabled us to examine the hydrodynamic parameters under a ligand-free condition. The nuclear extract was first fractionated by sucrose density gradient and gel filtration chromatography. ER was then immobilized on to HAP and incubated with [^{3}H]E_2 ± unlabeled DES as described in Experimental. ER was found to sediment at 6.0 S (6.01 + 0.04; mean + SE (n = 5)) in the gradient containing 0.4 M KCl and 10 mM Na_2MoO_4 (Fig. 3A). Enzymeimmunoassay for ER gave the similar sedimentation profile, indicating that this unoccupied ERn has the epitope as a putative ER. When the receptor–E_2 complexes were preformed in intact cells and then extracted with 0.4 M KCl, similar sedimentation profile was obtained under a high salt condition (Fig. 3B). In gel filtration chromatography using Sephacryl S-300, its Stokes radius was estimated as 5.5 nm (Fig. 4). ER precomplexed with [^{3}H]E_2 was found to have a similar Stokes radius (data not shown). Calculated molecular weight from these values (according to Siegel and Monty equation [21]) was 140,000 Da. This molecular weight was larger than that proposed for a mouse ER monomer [22]. Physicochemical parameters were also determined for the ERn solubilized by DNaseI. They migrated at 4.6S under high salt condition. In gel filtration chromatography, they eluted more slowly than KCl extracted ER. The estimated Stokes radius was 3.3 nm, and the molecular weight was calculated as 65,000 Da. This value was in a good accordance with the molecular weight of ER monomer which had been reported [22].

To estimate the molecular weight of ER monomer in B-1 cells, B-1 cells were incubated with 10 nM [^{3}H]TA for 1 h at 37°C. Then [^{3}H]TA–ER complexes were extracted from nuclei with 0.4 M KCl and applied to SDS–PAGE under a reduced condition. The radioactive band was detected at the molecular weight of 65,000 Da (Fig. 5A). Taken together, KCl extracted ERn seemed to exist as a oligomer form.

E_2-dependent gene activation

To examine the function of this unoccupied ERn, CAT activity assays were performed. A reporter plasmid, ERE-tk-CAT, was constructed and transfected into B-1 cells by the electroporation method. These cells were treated with E_2 and OHT for 48 h. CAT enzyme activity was enhanced by the addition of E_2 (Fig. 5B). The densitometric analyses revealed that E_2 (10^{-8} M) stimulation caused a 4.2 ± 1.5 (n = 3)-fold increase in CAT activity over that in nonstimulated (control) cells. This E_2-dependent enhancement was inhibited to the control level by the simultaneous addition of 10^{-7} M OHT. OHT alone did not show the agonistic activity. CAT assay indicated that unoccupied ERn in B-1 cells did not exhibit full biological function.

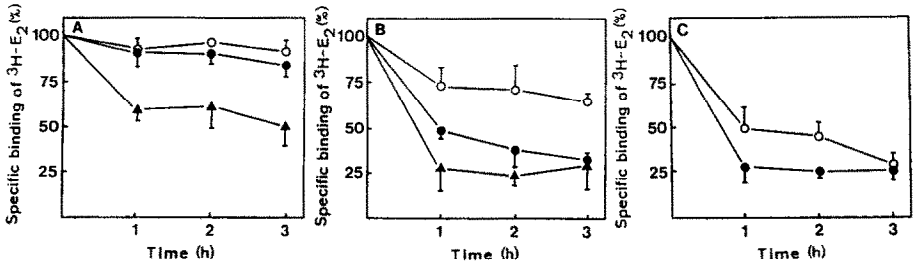

Fig. 2. Thermostability of unoccupied ER in each fraction. KCl-extracted nuclear ER(A), cytosol ER(B) and DNase I-solubilized nuclear ER(C) were immobilized on to HAP, and incubated at 30°C (○——○), 37°C (●——●) or 45°C (▲——▲) for 1–3 h in the absence of ligand. Then, the binding assay was performed at 4°C for 18 h. Specific [^{3}H]E_2 binding compared with that was determined without preincubation (100%). Results were expressed as mean ± SE of three separate experiments.

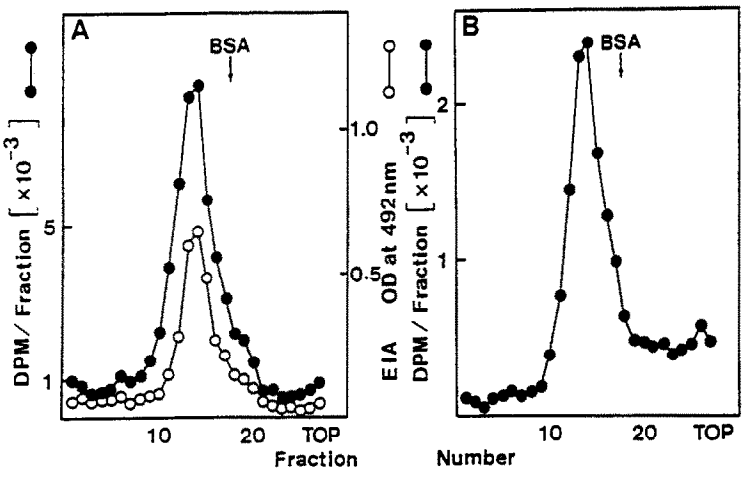

Fig. 3. Sedimentation profiles of KCl-extracted nuclear ER in B-1 cells. The nuclear extracts were prepared from B-1 cells untreated (panel A) or treated with 5 nM [^{3}H]E_2 at 37°C for 1 h (panel B). These extracts were applied to high salt sucrose density gradients. After centrifugation and fractionation, each fraction was mixed with 50 μl of HAP slurry. In the experiment described in Panel B, the radioactivity associated with the washed HAP was directly measured. In the experiments described in Panel A, the washed HAP was incubated with 5 nM [^{3}H]E_2 at 4°C for 18 h. After incubation, HAP was again washed to obtain the amount of [^{3}H]E_2 association with HAP. The nonspecific [^{3}H]E_2 binding data were not included into these panels since their values were always negligible. The aliquots of each fraction were also subjected to EIA (○——○ in panel A).

DISCUSSION

The present study reported herein clearly shows that ER present in B-1 cells is predominantly located in the nuclear fraction as the unoccupied form. The subcellular localization of unoccupied ER is still a matter to debate. Many immunocytochemical studies have shown that ER is located in the nucleus all times [4]. Even if the immunocytochemical procedures were employed, however, Parikh *et al.* have proposed that ER exist in a dynamic equilibrium among the plasma membrane, cytoplasm and nucleus [10]. On the other hand, the biochemical procedures usually reveal the cytoplasmic localization of unoccupied ER. These reported results are in contrast with the present observation. The experimental procedures described here were designed to minimize the artificial redistribution of ER. Phenol Red, which has been shown to cause the nucelar localization of ER in MCF-7 cells [23], was eliminated from culture

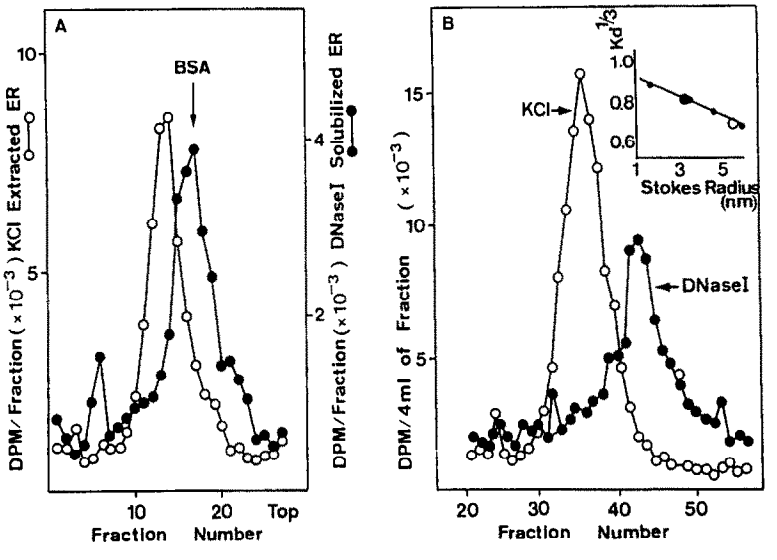

Fig. 4. Physicochemical parameters of ERn. Nuclear pellets of B-1 cells were extracted with 0.4 M KCl and treated with DNaseI (500 μg/ml) at 4°C for 1 h. The supernatant obtained by the centrifugation (10,000 *g* for 10 min) was served to sucrose gradient analysis (A) and gel filtration chromatography (B) under high salt condition. The relationship between the destribution coefficiency and Stokes radius was depicted in the insert of Panel B. (○——○) KCl-extracted ER, (●——●) DNaseI-solubilized ER.

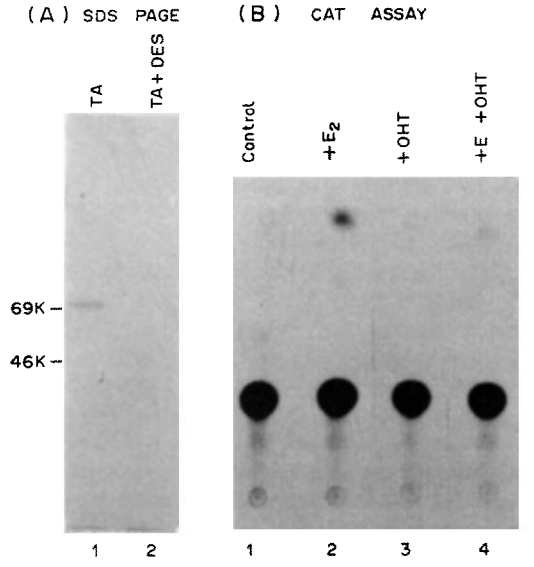

Fig. 5. SDS–PAGE of ERn and its biological activity. (A) B-1 cells were incubated with 10 nM [^{3}H]TA ± 1 μM radioinert DES at 37°C for 1 h and then subjected to the subcellular fractionation. The nuclear extract was analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. (B) B-1 cells were transfected with a reporter plasmid, ERE-tk-CAT. Transfected cells were cultured with 10^{-8} M E_2 (lane 2), 10^{-7} M 4-hydroxytamoxifen (lane 3), 10^{-8} M E_2 plus 10^{-7} M 4-hydroxytamoxifen (lane 4) or without hormone (lane 1) in Medium-II for 48 h. Then, cell extract was prepared and assayed for CAT enzyme activity using 0.1 μCi [^{14}C]chloramphenicol. Acetylated and non-acetylated forms of [^{14}C]chloramphenicol were separated by thin-layer chromatography and visualized by autoradiography.

media, although the subcellular localization of ER in B-1 cells was found to be unaffected by Phenol Red. Molybdate, which has been known to inhibit the transformation of ER [24], was included in the buffers for the subcellular fractionation.

A major implication of this work addresses the role of ligands for a formation of transformed ER. To our knowledge, this seems to be the first report showing the presence of unoccupied ERn with oligometric structure in a high salt condition. The hydrodynamic parameters of this ERn were similar to those of putative transformed ER complexed with E_2, eliminating the possibility that nonspecific aggregation of ERn occurs during the subcellular fractionation [25]. The molecular mechanism of ER transformation remains to be elucidated. Transformation of ER to so-called 5S form has been proposed to occur as a consequence of a homodimer formation in a E_2-dependent manner [8, 9]. The other possibility has been proposed that ER activation factor with a molecular weight of 62,000 exist as one component of transformed ER [26]. Nevertheless, the preformation of E_2–ER complexes has been considered to be a prerequisite to be converted from nontransformed to transformed state. However, the evidence described in this communication has demonstrated that unoccupied but transformed ER could exist in certain cells such as B-1 cells.

The unique characteristics of this unoccupied but transformed ER were identified. In general, ERn has been considered to be more thermostable than ERc (cytosolic ER) [27]. However, this unoccupied ERn in B-1 cells is extraordinarily heat-stable. The extensive digestion of nuclei with DNase I which has been known to release a "4S" form of [^{3}H]E_2–ER complexes from nuclei [28], abolished this thermal stability, concomitant with its molecular mass reduction to 65,000. These results suggest that this transformed ER identified in B-1 cells is extremely thermostable due to its oligometric structure.

The most important issue to be clarified is whether or not this transformed but unoccupied ER has the ability to activate gene expression. Our present observation suggests that transformation may be obligatory but not enough for ER to be converted into the biologically active state. Recently, the role of phosphorylation–dephosphorylation [29] or oxidation-reduction reaction [30] for the steroid receptor "activation" has been proposed. The molecular heterogeneity of ERn monomer in murine uteri has also become evident [31]. The relationship between the molecular state and its biological activity seem to be unclear. In order to discuss the process of transformed ER to the biologically active ER induced by complexing with E_2, this ERn in B-1 cells would provide us with the excellent model.

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