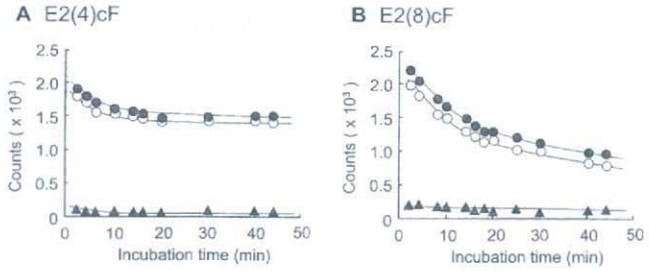

Fig. 3. **Change in the fluorescence intensity of fluorescent ligands E2(4)cF and E2(8)cF in the presence and absence of unlabelled estrogen.** E2(4)cF (A) and E2(8)cF (B) (100 nM) were incubated with ER (10 nM) at room temperature for 1 h. Total binding (filled circle) and non-specific binding (filled triangle) were obtained by the incubation with and without 17β-estradiol (E2: 10 μM), respectively. Specific binding (open circle) was estimated by subtraction of non-specific binding from total binding. The reaction mixture was treated with the same volume of 0.4% (w/w) dextran-coated charcoal in ice-cold 0.2 M PBS in order to remove unbound free fluorescent ligand. After incubation for 10 min, the mixture was centrifuged at 3,000*g* for 10 min at 4°C. The fluorescence intensity was measured for 200 μl supernatant aliquots transferred into 96-well polystyrene plates. After excitation at 485 nm (excitation energy: 20,000), the fluorescence intensity was monitored at 535 nm (counting time: 2 s) on a plate reader.

Table 5. **Binding characteristics of fluorescent ligands E2(n)cF to the estrogen receptor.**

E2(n)cF	Fluorescence intensities		
	Total binding	Non-specific binding	Specific binding
E2(2)cF	338 ± 41	101 ± 38	338 ± 14
E2(4)cF	1450 ± 77	178 ± 86	1280 ± 45
E2(6)cF	1020 ± 16	309 ± 88	713 ± 74
E2(8)cF	1980 ± 110	476 ± 18	1500 ± 97
E2(10)cF	522 ± 57	374 ± 32	148 ± 36
E2(12)cF	657 ± 120	155 ± 20	502 ± 99

The fluorescence intensity of E2(*n*)cF was measured in the presence or absence of the estrogen receptor ERα-LBD (10 nM in final). Receptor-unbound free ligand molecules were absorbed and removed by incubating with 0.4% dextran-coated charcoal. All experiments were performed on a 96-well polypropylene plate, and the solution contained 0.5 mg/ml bovine γ-globulin to prevent adsorption of compounds to the plate wall. Plate centrifugation was carried out at 4°C for 10 min over 3,000*g* on a plate rotor.

stirred for at least 1 h in PBS to get a well-swelled preparation. If not, it was not possible to obtain reproducible assay results, probably due to the insufficient and insecure adsorption capabilities of charcoal.

One of the essential conditions for the tracer in the receptor-binding assay is that it exhibit specific binding. Thus, all E2(*n*)cF were tested to determine their specific binding under the best assay conditions. Each E2(*n*)cF (100 nM in final) was incubated with the ER in the presence and absence of 17β-estradiol (10 μM) to ascertain the amounts of non-specific binding and total binding, respectively. Subtraction of the non-specific binding from the total binding affords the specific binding, and the results are summarized in Table 5. It is evident that E2(4)cF and E2(8)cF reveal sufficiently large specific

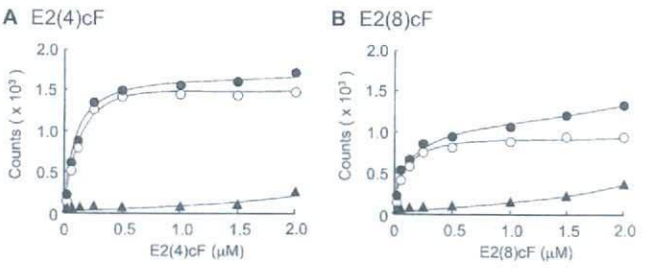

Fig. 4. **Saturation binding of fluorescent ligands E2(4)cF and E2(8)cF to ER.** Fluorescent ligands E2(4)cF (A) and E2(8)cF (B) were incubated with ER (10 nM) at room temperature for 1 h. Total binding (filled circle) and non-specific binding (filled triangle) were obtained by the incubation with and without 17β-estradiol (E2: 10 μM), respectively. Specific binding (open circle) was estimated by subtraction of non-specific binding from total binding. The reaction mixture was treated with the same volume of 0.4% (w/w) dextran-coated charcoal in ice-cold 0.2 M PBS in order to remove unbound free fluorescent ligand. After incubation for 10 min, the mixture was centrifuged at 3,000*g* for 10 min at 4°C. The fluorescence intensity was measured for 200 μl supernatant aliquots transferred into 96-well polystyrene plates. After excitation at 485 nm (excitation energy: 40,000), the fluorescence intensity was monitored at 535 nm (counting time: 3 s) on a plate reader.

bindings, which are 2–10 times higher than those of other fluorescent derivatives of E2(*n*)cF. We therefore decided to use both E2(4)cF and E2(8)cF as tracers in a novel fluorescence receptor-binding assay.

The specific binding of E2(4)cF and E2(8)cF to the estrogen receptor ERα-LBD in the presence of unlabelled 17β-estradiol (10 μM) was investigated by saturation binding. Figure 4 shows the saturation binding analysis of the binding of these fluorescent ligands to ERα-LBD. Under the same assay conditions, E2(4)cF and E2(8)cF exhibited binding profiles specific for each binding characteristic. It should be noted that the fluorescence intensity in saturation of E2(4)cF is considerably higher (about 2-fold) than that of E2(8)cF, suggesting that E2(4)cF is in a condition more restricted than E2(8)cF (Fig. 4). The cF moiety in E2(4)cF appears to be in firm and rigid surroundings that fix its molecular motion. It was concluded that the specific bindings of both E2(4)cF and E2(8)cF are sufficiently to construct a binding assay.

Scatchard plot analyses have demonstrated that the recombinant ERα-LBD shows a single binding mode (Fig. 5). Estimated B_{max} for E2(4)cF and E2(8)cF are 15.1 nmol/mg protein and 16.3 nmol/mg protein, respectively. These values are very compatible with the calculated value (16 nmol/mg protein) for the GST-fused ERα-LBD (molecular weight, 61,000).

Specific Emission Spectra of E2(4 or 8)cF with Estrogen Receptor—In the fluorescence spectra, we see two peaks at different wavelengths, namely, the excitation wavelength and the emission wavelength. At these peaks or spectra, we observed the increment of fluorescence intensity of E2(4)cF. Figure 6A shows the emission spectra of E2(4)cF in the presence and absence of ER in an aqueous buffer. The fluorescence intensity at the excitation wavelength (498 nm) by binding to the receptor increased only slightly (12%) over the initial value with a $\frac{1}{5}$-fold amount of receptor. The increase became greater

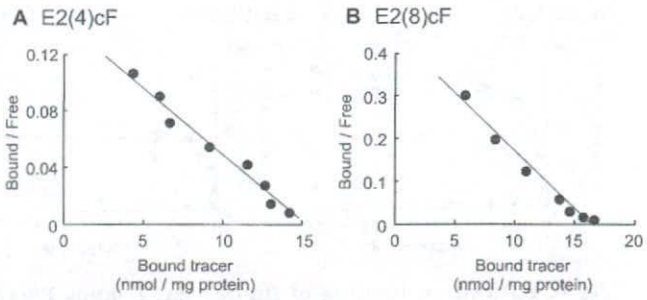

Fig. 5. **Scatchard analysis of saturation binding of fluorescent ligands E2(4)cF and E2(8)cF to ER.** The horizontal axis is the concentration (nmol/mg protein) of bound fluorescent ligands E2(4)cF (A) and E2(8)cF (B) to ER. The vertical axis is the ratio of the concentration of bound fluorescent ligand against free ligand to ER. (A) E2(4)cF; B_{max} (15.1 nmol/mg protein), K_d (104 nM). (B) E2(8)cF; B_{max} (16.3 nmol/mg protein), K_d (37.2 nM).

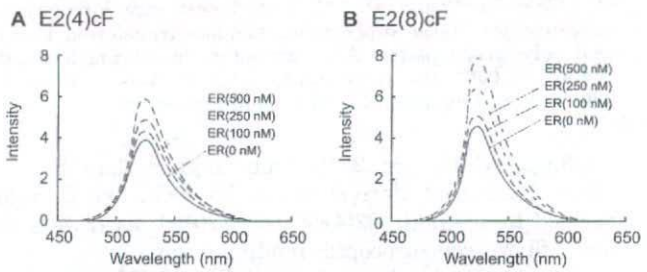

Fig. 6. **Fluorescence spectra of E2(4)cF and E2(8)cF in the presence or absence of ER.** For E2(4)cF (A) or E2(8)cF (B) (500 nM each), ER of 100, 250 and 500 nM concentrations were added at room temperature. Emission spectra were observed by excitation at 498 nm. Excitation at 485 nm afforded almost the same spectra. The results of the emission spectra are shown for solo 500 nM E2(4)cF (straight line), 500 nM E2(4)cF with 100, 250 and 500 nM ER (dashed line).

with increasing amounts of receptor; i.e. 25% with $\frac{1}{2}$-fold, 67% with equivalent amounts of receptor (data not shown). The peak maximum remained unchanged (λ_{ex} = 498 nm). When excited at 498 nm, the fluorescence intensity at the emission wavelength also increased to 16% of the initial value with a $\frac{1}{5}$-fold amount of receptor. The increment was enhanced by increasing the amount of receptor; i.e. 37% with $\frac{1}{2}$-fold, 62% with equivalent amounts of receptor. No shift in the peak maximum was observed (λ_{em} = 520 nm). When excited at 485 nm, which is the wavelength set on a plate reader, the fluorescence intensity at the emission wavelength (λ_{em} = 520 nm) increased to 16% of the initial value with a $\frac{1}{5}$-fold amount of receptor. The increment was enhanced by increasing the amount of receptor; i.e. 26% with $\frac{1}{2}$-fold, 48% with equivalent amounts of receptor. When E2(8)cF was examined for its emission spectra in the presence of the ER, it exhibited very enhanced increments in the fluorescence intensities (Fig. 6B). For instance, with the excitation at 485 nm, E2(8)cF showed a 96% increment with an equivalent amount of receptor, which is twice as large as that of E2(4)cF.

Receptor-binding Assay Using Fluorescent Tracers— Using E2(4)cF as a tracer, the receptor-binding assay was

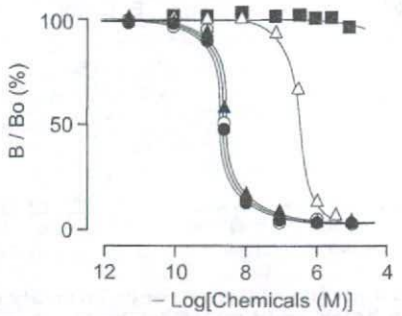

Fig. 7. **Concentration-dependent curves in a competition binding assay using fluorescent ligand E2(4)cF as a tracer for ER.** To a solution of ER (10 nM) was added fluorescent tracer E2(4)cF (5 nM) and compounds of 17β-estradiol (filled circle), estriol (open circle), estrone (filled triangle), 4-nonylphanol (open triangle) and fluorescein (filled square), respectively. The reaction mixture was incubated at room temperature for 1 h. The reaction mixture was treated with the same volume of 0.04% (w/w) dextran-coated charcoal in ice-cold 0.2 M PBS in order to remove unbound free fluorescent ligand. After incubation for 10 min, the mixture was centrifuged at 3,000*g* for 10 min at 4°C. The fluorescence intensity was measured for 200 μl supernatant aliquots transferred into 96-well polystyrene plates. After excitation at 485 nm (excitation energy: 40,000), the fluorescence intensity was monitored at 535 nm (counting time: 3 s) on a plate reader.

carried out essentially as performed in the assay using radiolabelled [^{3}H]17β-estradiol. The concentrations of estrogen receptor ERα-LBD (10 nM) and dextran-coated charcoal (0.04%) for the B/F separation were eventually determined to optimize the assay conditions to elicit a reasonable binding efficacy of fluorescent ligand E2(4)cF (5 nM). As shown in Fig. 7, standard estrogens such as estrone (E1), 17β-estradiol (E2) and estriol (E3) exhibited ideal dose-response curves with a high ability to displace E2(4)cF. Their IC_{50} values are compatible with those obtained from the binding assay using [^{3}H]17β-estradiol (Table 6).

As shown in the radiolabel binding assay, cF, a fluorophore of E2(4)cF and E2(8)cF, was completely inactive in both receptor-binding assays, indicating that fluorescein cannot displace estradiol in the estrogen-binding site. This result also implies that the putative interaction of the fluorophore of E2(4)cF and E2(8)cF with the receptor is extremely weak as compared with the specific interaction of the estrogen moiety. All these results clearly reveal that fluorescent ligands E2(4)cF and E2(8)cF possess essential structural and kinetic characteristics as a probe or tracer of the ER. This was also proven by the assay for a series of naturally occurring steroid hormones and the derivatives of estradiol (Table 6).

Diethylstilbestrol (so-called DES), a highly active alternative of 17β-estradiol, was also equipotent in both assays; i.e. 1.5–1.9 nM in the fluorescent binding assay and 1.8 nM in the radiolabel-binding assay (Table 7). Also, 4-hydroxytamoxifen, an antagonist of 17β-estradiol, was highly potent; i.e. 3.1–3.3 nM in the fluorescent binding assay and 3.1 nM in the radiolabelled binding assay. These results clearly indicate that ordinary aromatic organic compounds are able to displace fluorescent ligands E2(4)cF and E2(8)cF as well as steroids.

Table 6. **Binding activity of naturally occurring steroid hormones and derivatives of estradiol to estrogen receptor with fluorescence and radiolabeled tracers.**

Chemicals	IC_{50} (nM)		
	E2(4)cF	E2(8)cF	[^{3}H]17β-estradiol
Estrone (E1)	2.61 ± 0.23	2.57 ± 0.15	2.98 ± 0.23
17β-estradiol (E2)	1.13 ± 0.11	1.13 ± 0.13	1.18 ± 0.11
Estriol (E3)	3.67 ± 0.39	3.72 ± 0.69	3.44 ± 0.13
17α-ethinylestradiol	1.63 ± 0.10	1.72 ± 0.10	1.25 ± 0.11
17α-estradiol	4.09 ± 0.30	4.07 ± 0.13	4.02 ± 0.13
Testosterone	NB	NB	NB
Progesterone	NB	NB	NB
Carboxyfluorescein	NB	NB	NB

NB, not bound, implies that the chemical is not bound to the receptor at its 10 μM concentration.

Table 7. **Binding activity of various chemicals to estrogen receptor with fluorescence and radiolabeled tracers.**

Chemicals	IC_{50} (nM)		
	E2(4)cF	E2(8)cF	[^{3}H]17β-estradiol
17β-estradiol (E2)	1.13 ± 0.11	1.13 ± 0.13	1.18 ± 0.11
Diethylstilbestrol	1.53 ± 0.13	1.89 ± 0.13	1.80 ± 0.72
4-Hydroxytamoxifen	2.14 ± 0.27	2.58 ± 0.56	2.29 ± 0.49
4-Nonylphenol	1380 ± 191	1350 ± 185	1440 ± 176
n-Nonylphenol	2510 ± 566	2240 ± 730	ND
Bisphenol A	1050 ± 243	1090 ± 225	1160 ± 110
4-Octylphenol	2290 ± 166	2120 ± 427	ND
t-Octylphenol	4040 ± 524	4760 ± 647	ND
4-Cyclohexylphenol	700 ± 165	642 ± 234	ND
Diethylphthalate	NB	NB	NB
Di-*n*-butylphthalate	NB	NB	NB
Tributyltin chloride	NB	NB	NB
Triphenyltin Chloride	NB	NB	NB

ND not determined, implies that the IC_{50} (nM) value was not determined because of very weak receptor-binding affinity at its 10 μM concentration. NB not bound, implies that the chemical was almost completely inactive with no binding at its 10 μM concentration.

On the other hand, 4-nonylphenol, a putative endocrine disruptor for the ER, was only moderately active (IC_{50} = 1.35–1.38 μM). This result is also comparable to that obtained from the radiolabel-binding assay (1.44 μM) (Table 7). Similarly, bisphenol A, currently the most notable endocrine disruptor claimed to have the low-dose effects, was almost equipotent in both assays; i.e. 1.04–1.09 μM in the fluorescent-binding assay and 1.05 μM in the radiolabelled binding assay. Here, it is definite that bisphenol A is very weak to interact with ERα. Various candidate chemicals as endocrine disrupting chemicals were also examined in these three assay systems (Table 7). Tri-*n*-butyltin chloride, triphenyltin hydroxide and all phthalates were confirmed to be inactive for ERα.

It should be noted that the assay using E2(8)cF as a tracer afforded almost the same binding results as observed in the assay with E2(4)cF (Tables 6 and 7). The assay *per se* was performed under the same experimental conditions as those for E2(4)cF (Fig. 6), producing ideal dose–response curves, as seen in Fig. 6 for standard estrogens E1, E2 and E3. In conclusion, it is definitely worthwhile to use E2(4)cF and E2(8)cF as fluorescence labels in the binding assay for the ER.

DISCUSSION

Fluorescent Tracer with Cross-linker Between Estrogen and Fluorophore—In this study, we established a novel binding assay method, in which the specific binding was determined by a direct measure of fluorescence intensities. This is the first example of a binding assay utilizing a fluorescent tracer to measure the fluorescence intensity for the NRs. The results clearly indicate that an assay system using either E2(4)cF or E2(8)cF is adequate for assessing the ability of compounds to displace these tracers. Assay data are compatible with those obtained from the ordinary radiolabel binding assay using [^{3}H]17β-estradiol.

A receptor-binding assay for measuring the fluorescence intensity has been reported for G protein-coupled receptors such as seletonin $5HT_3$ receptor. However, selection or optimization of tracer ligands to deduce the maximal condition for measurement of fluorescence intensity was not carried out in this case. There is a similar case for assays utilizing fluorescence polarization. The pharmacophores were cross-linked to the fluorophores with just a single cross-linker. The present results clearly show that the fluorescein characteristics of E2(*n*)cF are dependent upon the varying cross-linking methylene chain lengths, and are able to be optimized.

The fact that changes in the fluorescence intensity upon specific binding to the receptor depend upon the methylene lengths of cross-linkers between 17α-ethinylestradiol and cF indicates that the interaction of fluorescein with the receptor can be substantiated only at acceptable sites at a proper distance. This was clearly shown by the ordinary receptor-binding assay, in which E2(8)cF afforded the best binding affinity to the receptor among a series of E2(*n*)cF. Although E2(8)cF is still ~100 times less potent than 17β-estradiol itself, its binding specificity is sufficient to estimate the ability of compounds to displace it at the binding site. E2(4)cF, which is ~10 times less potent than E2(8)cF in displacing [^{3}H]17β-estradiol, exhibited somewhat larger changes in the fluorescence intensity upon specific binding to the receptor. This result may imply that the cF moiety is in a structurally restricted circumstance due to its shorter (by approximately half) cross-linker.

In the assay methods to measure the fluorescence polarization, two different types of fluorescent ligands were used. One of these fluorophores was fluormone ES1, a structurally modified diethylstilbestrol (DES). ES1 is an intrinsically fluorescent non-steroid estrogen and exhibits a short excitation wavelength (360 nm), providing a weak fluorescence at the emission wavelength (530 nm). Since many samples of interest such as biological fluids often contain adventitious fluorescence, a short excitation wavelength seriously raises the background against which the measurement must be made. Another fluorophore reported is FITC, fluorescein isothiocyanate, in which fluorescein exhibits a rather long excitation (490 nm) and emission (520 nm). However, synthesized 17α-substituted E2 derivative has a much shorter cross-linker that corresponds to E2(0)cF. A short spacer might cause steric hindrance between estradiol and fluorescein to interact with the receptor-binding site, although their binding affinity was not evaluated in the regular

radioligand-binding assay. The present study clearly shows that fluorescent tracers should be optimized for their cross-linker between the pharmacophore and the fluorophore.

Specific Interaction of Fluorescein with Estrogen Receptor—Changes in the fluorescence intensity depend upon specific binding to the receptor. The increase in fluorescence intensity of E2(4)cF at the emission wavelength in the presence of estrogen receptor ER-LBD is due to its binding to the receptor. Since the increase is dependent upon the concentration of receptor, the characteristic changes in the fluorescence intensity are due to the interaction between the fluorophore and the receptor protein. This specific interaction may result in a freeze in movement of the fluorophore, cF.

E2(8)cF exhibited much enhanced increments in fluorescence intensities in its emission spectra in the presence of the ER. As indicated, it showed a 96% increment with an equivalent amount of receptor, which is twice as large as that of E2(4)cF. This result appears to disagree with the result obtained from the saturation binding analysis. The fluorescence intensity of E2(8)cF in saturation analysis was definitely smaller than that of E2(4)cF (Fig. 4). As shown in Table 4, the receptor-binding affinity of E2(8)cF is almost 10 times higher than that of E2(4)cF. Thus, if we add the same concentration of the receptor ER, ER would bind much more abundantly E2(8)cF than E2(4)cF. This would make the fluorescent intensity of E2(8)cF stronger than that of E2(4)cF as in Fig. 6. It should be noted that the saturation binding analysis was performed under the condition of the charcoal treatment for B/F separation, while the examination was carried out with no charcoal. Charcoal may remove receptor-unbound ligands and affect the receptor–ligand equilibrium. Such a removal would be more effective for E2(8)cF than for E2(4)cF, because E2(8)cF has a hydrophobic methylene chain twice as long as that of E2(4)cF.

Both E2(4)cF and E2(8)cF are very unique in having a rather long cross-linking spacer between estrogen and fluorescein. Flexibility due to attachment of the fluorophore may cause a so-called propeller effect, resulting in depolarization to bring out no changes in the fluorescence intensity. To minimize this propeller effect, the molecular flexibility should be diminished by the 'specific' interaction of the fluorophore with the receptor. Based on this rationale, fluorophore cF should stay at this certain 'specific' receptor site, presumably a highly hydrophobic location. This 'specific' binding would also diminish the flexibility of the methylene chain. To identify such a 'specific' binding site for the fluorophore, it is essential to determine the optimal chain length. To make such a determination, we prepared candidate compounds with a series of aliphatic polymethylene cross-linkers of varying chain lengths. In the preparation of tracers, it is now evident that optimization of the spacer structure, namely, the structure of a cross-linker between the fluorophore and the pharmacophore, is extremely important.

It was eventually found that E2(*n*)cF with tetramethylene $(CH_2)_4$ and octamethylene $(CH_2)_8$ exhibit much larger changes in fluorescence intensity. As such, cF, attached to 17α-ethinylcarboxyestradiol via polymethylene $(CH_2)_4$ in E2(4)cF and $(CH_2)_8$ in E2(8)cF, is captured at different sites of the ER-LBD. These sites are ~6 Å apart from each other, corresponding to the $(CH_2)_4$ chain length, the structural circumstances of which must be different, especially with regard to its fluorescent characteristics.

Optimization of Assay Conditions and Advantages of Assay—To analyse the precise interactions of cF in E2(*n*)cF, its adsorption to the assay plates should be prevented. In a preliminary stage of the experiment to set the assay conditions, we used BSA as a blocker that protects the tracer to adsorb to the polypropylene microwell plate surface. However, unstable and irreproducible results were obtained from the successive assays. When the fluorometry measurement was carried out for plates of exactly the same concentration, the fluorescence intensity was found to vary from plate to plate. These results suggest that the synthesized fluorescein-linked estrogen derivatives had a strong interaction with BSA, perturbing the fluorometry measurements. Indeed, several reports have shown the nonspecific adsorption of fluorescein to BSA (*42*, *43*). This issue is resolved only when BSA is replaced by bovine γ-globulin (*44*). After all, bovine γ-globulin has been found to afford stable and reproducible results in fluorometry measurements.

The present study provides for the first time a fluorescence receptor-binding assay that measures the changes in the fluorescence intensity. Although this method requires B/F separation of the tracer, treatment with dextran-coated charcoal gives a full separation. One of the greatest advantages of this method is the direct use of microwell plates of 96-holes for centrifugation followed by plate fluorometry measurements. Another advantage is that experimentation can be performed in an ordinary laboratory, with no special regulations, unlike the RI laboratory required for the radiolabel receptor-binding assay. The present method would afford a universal procedure to evaluate the binding affinity of NRs.

Bisphenol A is a Weak Binder of Estrogen Receptor—Bisphenol A is one of the highest volume chemicals produced worldwide as a starting material for polycarbonate plastics and epoxy resins. Long known as an estrogenic chemical, bisphenol A is suspected of interacting with human ER (*45*, *46*) or acting as an antagonist for a human androgen receptor (AR) (*47*). However, it has been notified that its binding to ER and AR and its hormonal activity are extremely weak. Indeed, in the present study, we demonstrated that the binding activity of bisphenol A is 700–800 times weaker than with natural hormone 17β-estradiol (Table 7). Based on the idea that bisphenol A may interact with NRs other than ER and AR, we screened a series of NRs and eventually explored ERRγ as the target receptor of bisphenol A (*9*, *11*).

Bisphenol A was found to bind strongly to ERRγ, one of 48 human NRs, with high constitutive basal activity (*9*). Bisphenol A's binding to ERRγ was further demonstrated by X-ray crystallographic analysis of the complex between bisphenol A and ERRγ (*10*). Whether or not bisphenol A is an endocrine disruptor that exhibits 'low-dose effects' has long been controversial, and there is a scientific debate over whether or not low BPA doses have reproductive and developmental effects in humans (*48–50*). To evaluate correctly the receptor-binding affinity is crucially important for appropriate interpretation of receptor responses

of the various compounds, and thus it is critical to develop the efficient assay systems.

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ERRγ tethers strongly bisphenol A and 4-α-cumylphenol in an induced-fit manner

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ABSTRACT

A receptor-binding assay and X-ray crystal structure analysis demonstrated that the endocrine disruptor bisphenol A (BPA) strongly binds to human estrogen-related receptor γ (ERRγ). BPA is well anchored to the ligand-binding pocket, forming hydrogen bonds with its two phenol-hydroxyl groups. In this study, we found that 4-α-cumylphenol lacking one of its phenol-hydroxyl groups also binds to ERRγ very strongly. The 2.0 Å crystal structure of the 4-α-cumylphenol/ERRγ complex clearly revealed that ERRγ's Leu345-β-isopropyl plays a role in the tight binding of 4-α-cumylphenol and BPA, rotating in a back-and-forth induced-fit manner.

Bisphenol A (BPA) has been recognized as one of the most potent endocrine disruptors, functioning even at very low doses. Although it is anticipated that estrogen receptor (ER) would mediate these effects of low-dose BPA, studies revealed that BPA bound to ER very weakly [1–5]. On the other hand, we have recently discovered that a human nuclear receptor, estrogen-related receptor γ (ERRγ), acts as a specific receptor for BPA (K_D = ca. 5.5 nM) [6,7]. ERRγ elicits a high basal constitutive activity with no ligand, and BPA was found to fully retain this activity. These findings necessitate that we reevaluate the low-dose effects of BPA in relation to the high-binding ability of BPA to ERRγ.

Recently, we successfully performed an X-ray analysis of the crystal structure of the complex formed between BPA and the ligand-binding domain (LBD) of ERRγ [8]. BPA, 2,2-bis(4-hydroxyphenyl)propane, was found in the binding pocket of ERRγ-LBD just like a tightly bound natural ligand. BPA-bound ERRγ maintains the activation conformation of authentic ERRγ, which helps to explain why the BPA-ERRγ complex retains a high basal constitutive activity.

In this study, we preliminarily examined the binding ability of 4-α-cumylphenol, which lacks one of the two phenol-hydroxyl groups of BPA (Fig. 1). Surprisingly, 4-α-cumylphenol was found to bind to ERRγ as potently as BPA. Since diphenylpropane with no phenol-hydroxyl group was completely inactive, the phenol-hydroxyl group of 4-α-cumylphenol is essential for the binding to ERRγ. This means that any compounds having the phenol group are potential candidates for strong binders of ERRγ.

One of the two phenol-hydroxyl groups of BPA simultaneously forms hydrogen bonds with Glu275 and Arg316, while the other makes a hydrogen bond with Asn346. The question of which hydrogen bond holds 4-α-cumylphenol in the ligand-binding pocket of ERRγ is crucial for predicting the binding potential of the phenol compounds. In addition, answering this question is crucial for elucidating the structural reason why 4-α-cumylphenol is as potent as BPA. In the present study, to answer to these questions, we analyzed the X-ray crystal structure of the complex of 4-α-cumylphenol/ERRγ. We succeeded in crystallizing the complex, and the X-ray analysis revealed a strong hydrogen bonding of the phenol-hydroxyl group with Glu275/Arg316 of ERRγ and a strong hydrophobic interaction between the phenyl group and the isopropyl group of Leu345.

Abbreviations: BPA, bisphenol A; CBB, Coomassie Brilliant Blue; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DES, diethylstilbestrol; ER, estrogen receptor; ERR, estrogen-related receptor; LBD, ligand-binding domain; 4-OHT, 4-hydroxytamoxifen; PCR, polymerase chain reaction.

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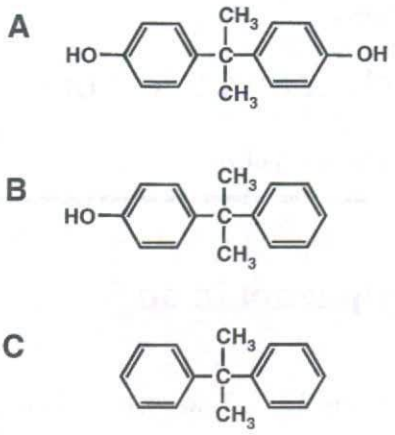

Fig. 1. Chemical structure of bisphenol A (BPA) and its derivatives. (A) BPA, (B) 4-α-cumylphenol, and (C) 2,2-diphenylpropane.

Materials and methods

Receptor protein expression and purification. Preparation of the receptor protein was carried out essentially as reported previously [8]. The cDNA fragment encoding human ERRγ-LBD (corresponding to amino acid residues 222–458) was generated by PCR, and the amplified product was cloned into the expression vector pGEX 6P-1 (Amersham Biosciences, Piscataway, NJ, USA) to express the product as a glutathione-*S*-transferase (GST) fusion protein by using *Escherichia coli* BL21 [6]. GST was cleaved by PreScission Protease (Amersham Biosciences), and the protein concentration was determined by the Bradford method [9].

Radio-ligand-binding assays. The receptor-binding assay was conducted essentially as reported previously [10] using [^{3}H]4-hydroxytamoxifen (4-OHT) (80 Ci/mmol) from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). To estimate the binding affinity, the IC_{50} values (the concentrations for the half-maximal inhibition) were calculated from the dose–response curves by using the nonlinear analysis program ALLFIT [11].

Luciferase reporter gene assay. A luciferase reporter gene assay using HeLa cells was carried out by the method of transient transfection as reported previously [7]. Luciferase activity was measured by using luciferase assay reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Crystallization of protein complex followed by X-ray data collection and processing. Purified ERRγ-LBD was concentrated by ultrafiltration. Co-crystallization with a threefold molar excess of 4-α-cumylphenol was carried out with the hanging drop vapor diffusion method as described [8]. X-ray diffraction data were collected at SPring-8 (Hyogo, Japan). The data were integrated and scaled using the HKL2000 package [12]. ERRγ-LBD with no ligand (apo-ERRγ-LBD) was obtained using essentially the same method as for the 4-α-cumylphenol/ERRγ-LBD complex. For data collection, crystals were transferred into a cryoprotectant solution containing 30% sucrose.

Structure determination and refinement. A monomer model of BPA/ERRγ-LBD (2E2R) was used as a search molecule for molecular replacement using MOLREP [13] in CCP4 [14]. The position of the 4-α-cumylphenol/ERRγ-LBD complex and apo-ERRγ-LBD in each asymmetric unit was located and the structure was refined at 2.0 and 1.8 Å, respectively, using REFMAC5 [15] in CCP4. Manual adjustment and rebuilding of the model including 4-α-cumylphenol and water molecules were performed using the program Coot [16].

The final 4-α-cumylphenol/ERRγ-LBD complex model contained residues 232-458 of ERRγ, one 4-α-cumylphenol, one glycerol, and 149 water molecules. The final apo-ERRγ-LBD model also contained residues 232–458 of ERRγ and 219 water molecules. The final models were validated with PROCHECK [17]. Atomic coordinates for the structure has been deposited in the Protein Data Bank with accession code 2ZAS for the 4-α-cumylphenol/ERRγ-LBD complex and 2ZBS for the apo form of ERRγ-LBD.

Results and discussion

The binding site of phenol-hydroxyl group of 4-α-cumylphenol in ERRγ

The competitive receptor-binding assay was performed using [^{3}H]4-OHT for GST-ERRγ-LBD. As shown in Table 1, it was found that BPA, 4-α-cumylphenol, and 4-OHT are almost equally potent, having similar IC_{50} values of approximately 10 nM. In contrast, 2,2-diphenylpropane was extremely weak (IC_{50} > 10,000 nM). Since 2,2-diphenylpropane has no phenol group (Fig. 1), it is clear that one of the phenol-hydroxyl groups of BPA is indispensable for the interaction with a binding pocket of ERRγ. These results were confirmed in a separate assay using [^{3}H]BPA [18]. It should be noted that 4-α-cumylphenol is as potent as BPA, although 4-α-cumylphenol lacks one of the two phenol-hydroxyl groups of BPA.

A major goal of the present study was to elucidate the phenol-hydroxyl groups shared by both BPA and 4-α-cumylphenol. In the case of BPA, we designated the phenol group bridged by hydrogen bonds to Glu275/Arg316 as the A ring, and the phenol group hydrogen-bonded to Asn346 as the B ring. Thus, one of the purposes of the present study was to determine whether 4-α-cumylphenol possesses the A ring or the B ring. We solved the crystal structure of ERRγ-LBD in complex with 4-α-cumylphenol at a resolution of 2.0 Å (space group $P4_12_12$) (Supplementary Table). The complex formed was crystallized in a homodimeric form using crystallographic 2-fold symmetry (Fig. 2A). A 4-α-cumylphenol molecule was defined very well in the complex (Fig. 2B).

For 4-α-cumylphenol, we simply found the A ring in the 4-α-cumylphenol/ERRγ- LBD complex. The solo phenol-hydroxyl group of 4-α-cumylphenol was involved in the hydrogen bonding with both Glu275 and Arg316. Superimposition of 4-α-cumylphenol and BPA (2E2R) [8] in the ERRγ-LBD complexes showed a conformational identity that could readily account for the similarity in binding modes to the binding pocket. The phenol A ring of BPA superimposed almost completely with the corresponding A ring of 4-α-cumylphenol.

We have recently reported the binding potentials between the phenol-hydroxyl group of BPA and ERRγ receptor residues Glu275 and Arg316 in the LBD [7]. Wild-type ERRγ-LBD showed a strong-binding ability (K_D = ca. 5.5 nM) for [^{3}H]BPA, but the simultaneous mutation to Ala at positions 275 and 316 resulted in an absolute inability to capture BPA. The ERRγ receptor appears to form an appropriate structure with the Glu275 and Arg316 residues, presumably to arrest the phenolic compound as an endogenous ligand.

Table 1
The receptor-binding affinity of chemicals in the assay using [^{3}H]4-OHT as a tracer for the human estrogen-related receptor γ (ERRγ)

Chemicals	Binding affinity (IC_{50}, nM)
Bisphenol A (BPA)	13.1 ± 2.34
4-Hydroxytamoxifen (4-OHT)	10.3 ± 0.80
4-α-Cumylphenol	13.9 ± 1.98
2,2-Diphenylpropane	Inactive

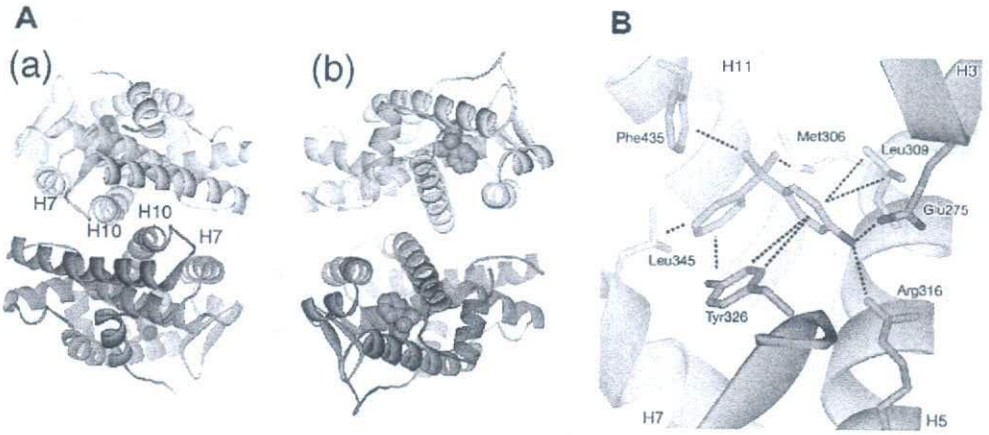

Fig. 2. Overall structure of the dimer of the 4-α-cumylphenol/ERRγ-LBD complex (A) and the complex structure between 4-α-cumylphenol and ERRγ-LBD (B). (A) The panoramic view of the whole-sphere 4-α-cumylphenol/ERRγ-LBD complex homodimer. Each panel shows the 3D-structure pictured from the top and the bottom with 180° rotation. One molecule of 4-α-cumylphenol (space-filling) is in a ERRγ-LBD molecule (ribbon). (B) The complex structure in which 4-α-cumylphenol is placed in the ligand-binding pocket of the ERRγ-LBD. The dotted lines show the hydrogen and the hydrophobic bonds.

Structural elements holding 4-α-cumylphenol in the ERRγ-LBD

4-α-Cumylphenol was found to be in a very prominent-binding site constructed of a series of amino acid residues in the ERRγ-LBD. The receptor residues within a range of 5 Å include Leu268, Cys269, Leu271, Ala272, and Glu275 from H3; Trp305, Met306, Leu309, Ile310, Val313, and Arg316 from H5; Tyr326 from β-strand 1 (S1); Leu342, Leu345, Asn346, and Ile349 from H7; Ala431 from H10; Phe435 from H11; and Phe450 from H12.

Tyr326 in S1 has been reported to interact with Asn346 (H7) through a hydrogen bond [19,20]. This hydrogen bond is also maintained in the 4-α-cumylphenol/ERRγ-LBD complex. It should be noted that Tyr326 is the major amino acid residue necessary for the placement of 4-α-cumylphenol in the ERRγ-LBD complex. As seen for BPA [8], the A ring of 4-α-cumylphenol is sandwiched by hydrophobic interactions between Tyr326 and Leu309 (Fig. 2B).

Tyr326 also keeps the cumyl-benzene ring of 4-α-cumylphenol by the OH/π interaction [21,22] (Fig. 2B). This cumyl-benzene ring also interacts with the isobutyl group of Leu345. Thus, the cumyl-benzene ring is in a binding site constructed by Tyr326 and Leu345. As to the two methyl groups on the sp^3-C atom, the one faces Phe435 (H11), and the other faces the Met306 sulfur atom with a non-covalent electron pair.

Conformation changes of ERRγ-Leu345-β-isobutyl by the back-and-forth rotation to receive either phenol or phenyl

Another purpose of this study was to determine why 4-α-cumylphenol exhibits very strong-binding activity in the manner of BPA. The reason was found be the back-and-forth rotation of the Leu345 residue (Fig. 3A), which causes the residue either to interact with the phenyl group of 4-α-cumylphenol or to avoid the phenol group (B ring) of BPA.

The phenol B ring of BPA is directed towards H7 to capture Asn346 by its hydrogen bond. Also, the benzene ring of the cumyl group ($-C(CH_3)_2-C_6H_5$), namely, the phenyl group of 4-α-cumylphenol, is directed towards Asn346. However, this benzene ring does not have a hydroxyl group, and thus there is no hydrogen bond between the benzene ring and Asn346. Usually, the lack of such a hydrogen bond greatly reduces the ability of the compound to bind to a receptor molecule, thereby weakening the receptor-binding affinity. However, 4-α-cumylphenol consistently exhibited the same strong binding to ERRγ. It was thus assumed that there must be a certain structural element of the ERRγ-LBD-binding pocket that stabilizes the binding of the cumyl-benzene ring. When we superimposed the 4-α-cumylphenol/ERRγ-LBD complex with the BPA/ERRγ-LBD complex, we found a very clear conformation difference of the isobutyl group of Leu345, as shown in Fig. 3A.

This Leu345-isobutyl group ($-_\beta CH_2-_\gamma CH(_\delta CH_3)_2$) was found to face the benzene ring of 4-α-cumylphenol in the 4-α-cumylphenol/ERRγ-LBD complex (Fig. 3A and B(a)). In this conformation, the distance between the carbon atom of either $_\gamma$CH or $(_\delta CH_3)_2$ and the *para*-carbon atom of the benzene ring is approximately 3.7 Å, and thus $_\gamma$CH and $(_\delta CH_3)_2$ undergo a so-called CH/π-type hydrophobic interaction with the benzene π-electrons. This interaction stabilizes the receptor binding of the cumyl-benzene ring.

In the BPA/ERRγ-LBD complex, however, the Leu345-isobutyl group was found to turn its back against the B ring of BPA (Fig. 3A and B(b)). The isobutyl group, $-_\beta CH_2-_\gamma CH(_\delta CH_3)_2$, attaches to the α carbon ($_\alpha$C) of Leu345, and this group can rotate freely around the $_\alpha C-_\beta C$ bond. It is clear that the isopropyl group rotates about 180° between these two complexes (Fig. 3A). In the BPA/ERRγ-LBD complex, Leu345-$_\beta CH_2-_\gamma CH(_\delta CH_3)_2$ faces its two methyl $(_\delta CH_3)_2$ groups ('back'-face) toward the phenol B ring of BPA. This creates an appropriate space for the phenol-hydroxyl group (Fig. 3B(b)), giving the hydroxyl group a chance to form a hydrogen bond with the adjacent Asn346 amide group, $-CH_2-CONH_2$.

The finding that Leu345-$_\gamma CH(_\delta CH_3)_2$ directly or indirectly contributes to the stable binding of 4-α-cumylphenol and BPA is of great interest. ERRγ-LBD appears to make the most of the small space by the back-and-forth rotations of the isobutyl group of Leu345, in order to place the phenol group (BPA) and the benzene ring (4-α-cumylphenol). Given that such a back-and-forth rotation was not feasible, BPA could not be held in this pocket. As shown in Fig. 3B(b'), when the Leu345-isobutyl group keeps its conformation, as in the 4-α-cumylphenol/ERRγ-LBD complex, the phenol B ring of BPA comes into complete collision with Leu345-$_\gamma CH(_\delta CH_3)_2$. Also, in the event that the Leu345-isobutyl group maintains its conformation, as in the case of the BPA/ERRγ-LBD complex, there is too large a space for the benzene ring of 4-α-cumylphenol and Leu345-$_\gamma CH(_\delta CH_3)_2$ to interact with each other, as shown in Fig. 3B(a').

Induced-fit binding of BPA and 4-α-cumylphenol to ERRγ

We also succeeded in analyzing the crystal structure of the apo form of ERRγ-LBD (PDB id code 2ZBS) as reported by Greschik et al. (1TFC and 1KV6) [19,20], and also by Wang et al. (2GP7) [23]. When the superimposition between ERRγ-LBD from the 4-α-cumylphenol/ERRγ-LBD complex and apo-ERRγ-LBD was

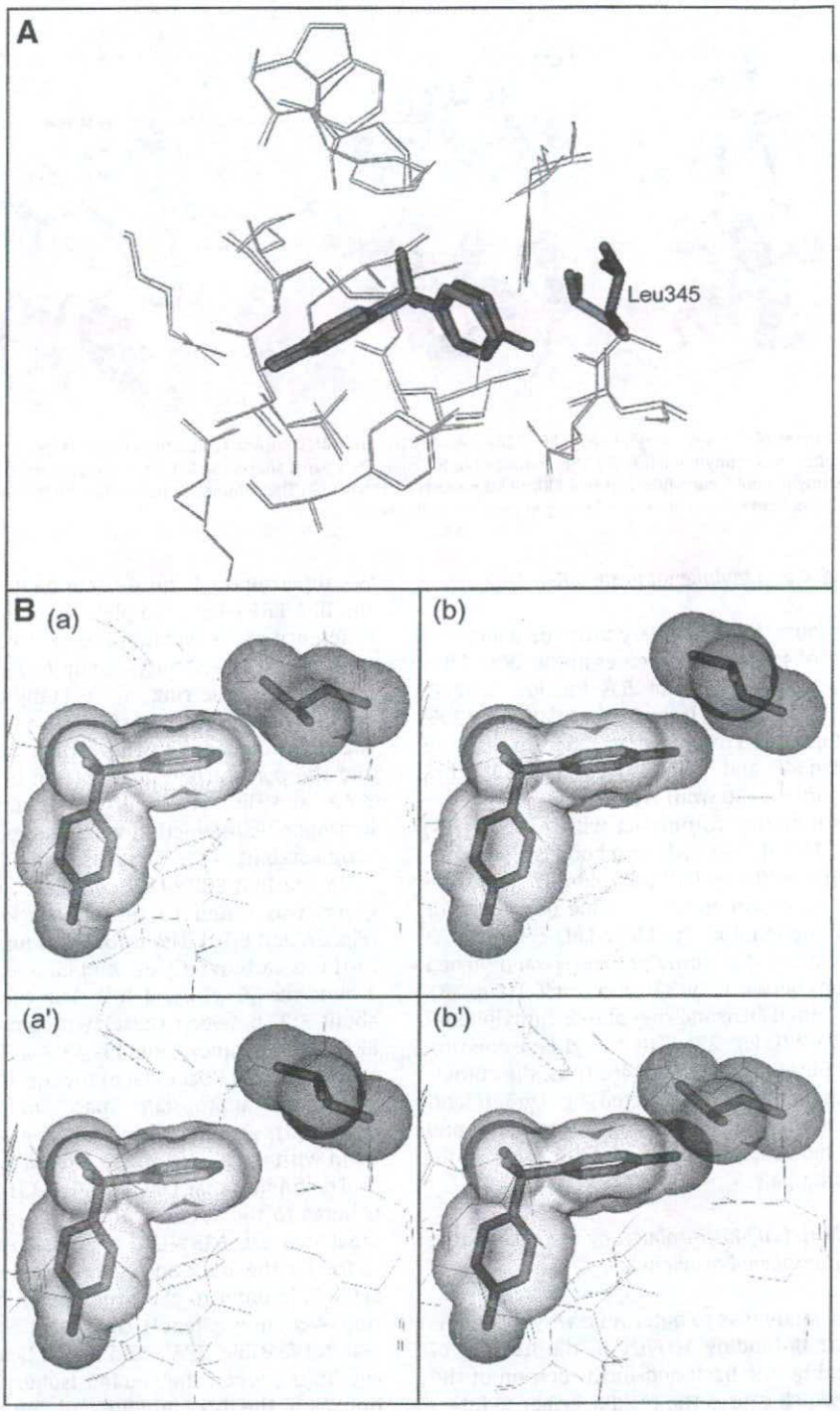

Fig. 3. Superimposition between the 4-α-cumylphenol/ERRγ-LBD and the BPA/ERRγ-LBD complexes. (A) The 4-α-cumylphenol/ERRγ-LBD complex (blue) (2ZAS) is superimposed with the BPA/ERRγ-LBD (green) (2ZBS). The amino acid residues shown are in close proximity to ligands within 5 Å. The isopropyl group ($-CH(CH_3)_2$) of Leu345 is in the shape of the letter Y. The back-face of Leu345-isopropyl of the 4-α-cumylphenol/ERRγ-LBD complex (blue) is directed towards the phenyl group of 4-α-cumylphenol (blue), whereas the forth-face of the BPA/ERRγ-LBD complex (green) is directed towards the phenyl group of BPA (green). (B) The back-and-forth rotation of Leu345 ensures the appropriate binding pocket for 4-α-cumylphenol (a and a') and BPA (b and b'). The surfaces based on the van der Waals radius of ligands and Leu345 are shown in each panel. (a) 4-α-Cumylphenol (blue) and Leu345 in the 4-α-cumylphenol/ERRγ-LBD complex (blue) maintain an appropriate space at the nearest C-C distance of approximately 3.7 Å. (a') The virtual complex between 4-α-cumylphenol (blue) and Leu345 in BPA/ERRγ-LBD (green) shows too large a space for mutual interaction. (b) BPA (green) and Leu345 in the BPA/ERRγ-LBD complex (green) maintain an appropriate distance of about 4.0 Å. (b') In the virtual complex between BPA (green) and Leu345 in 4-α-cumylphenol/ERRγ-LBD (blue), Leu345 comes into collision with the phenol-hydroxyl group of BPA.

carefully checked, the Leu345-isobutyl group was found to be in almost the same position (Fig. 4A). This implies that ERRγ originally keeps the Leu345 residue in the complex just as in apo-ERRγ-LBD, and thus 4-α-cumylphenol binds to a natural ERRγ. Turning to the binding of BPA to ERRγ, it is evident that Leu345 does make a rotation of about 180° to adopt the phenol group of BPA. If this rotation were not feasible, BPA should not have bound to ERRγ.

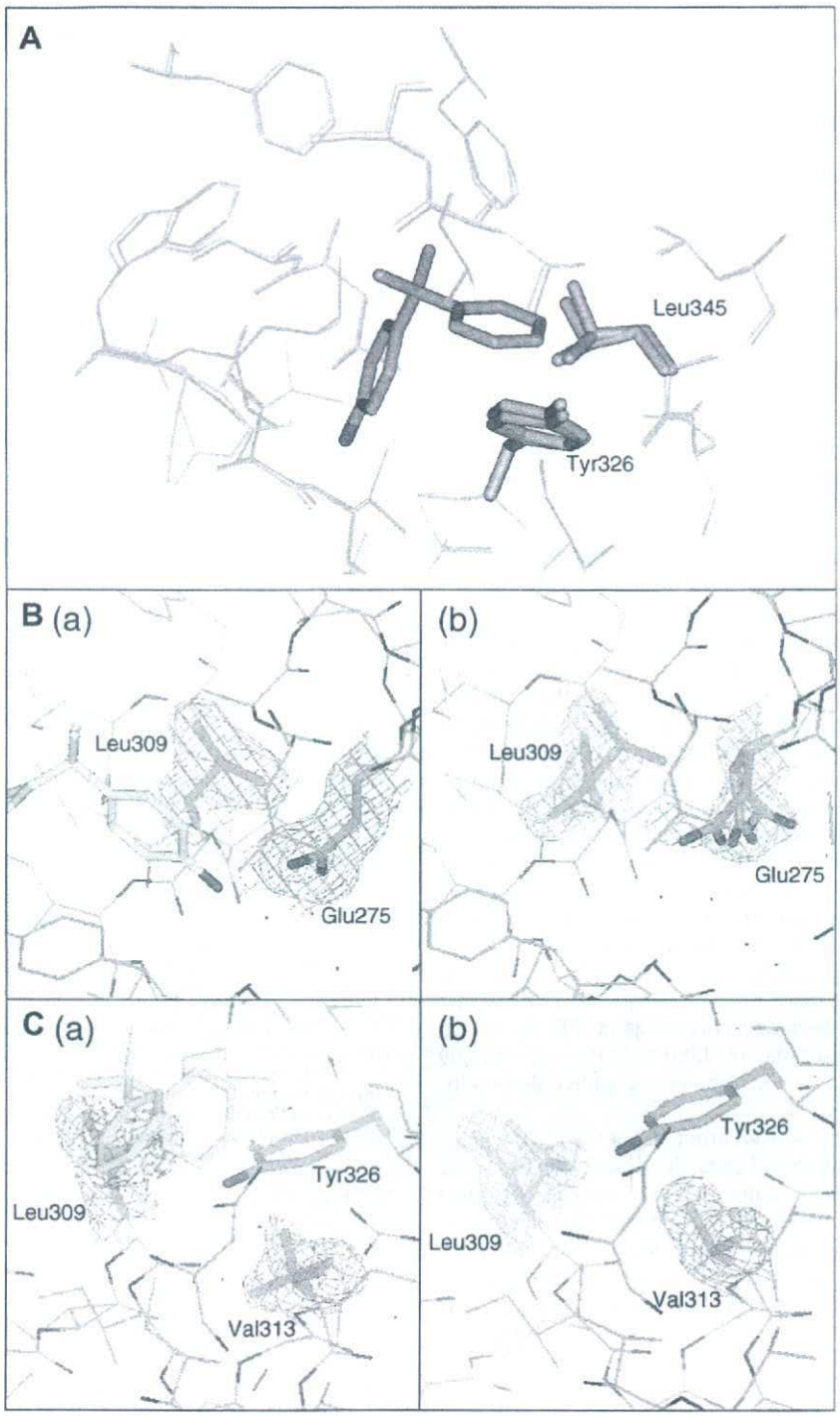

Fig. 4. Induced-fit binding of 4-α-cumylphenol to the ERRγ-LBD apo form. Superimposition of the 4-α-cumylphenol/ERRγ-LBD complex and apo-ERRγ-LBD. (A) The residues shown are in close proximity (within 5 Å) to 4-α-cumylphenol. The Leu345-isobutyl group and Tyr326-*para*-hydroxyl benzyl group are highlighted. (B) (a) Induced repositioning of Glu275 and Leu309 by the binding of 4-α-cumylphenol and (b) their conformationally released forms in the apo form. (C, a) In conjunction with the binding of 4-α-cumylphenol, Val313 is conformationally in the two different conformations, one of which is seen in the apo form (C, b). In the apo form, the Val313-isopropyl group is in the tight interaction with the Tyr326-phenol phenyl group. However, in the complex structure, the Tyr326-phenol ring tilts at the angle of about 15 degree (see Fig. 4A), reorienting Val313-isopropyl in the second position as seen in (C, a). The residues netted in blue are defined in a certain conformation, while those in magenta are in the mobile positions undefined or in a multiple conformations.

The side chain of Glu275 is not well defined in apo-ERRγ-LBD owing to its high mobility (Fig. 4B(b)). However, in the 4-α-cumylphenol complex, it reorients towards the hydroxyl group of the phenol A ring and is tightly positioned by the hydrogen bonding (Fig. 4B(a)). Similarly, the isobutyl side chain of Leu309 is first in the highly mobile position undefined in apo-ERRγ-LBD (Fig. 4B(b) and C(b)), while it reorients towards the phenol A benzene ring of 4-α-cumylphenol (Fig. 4B(a) and C(a)). By contrast, the side chain of Val313 became defined in the two different conformations in the 4-α-cumylphenol complexes (Fig. 4C(a)). This reorientation of Val313-isopropyl in the two different positions appears to be due to the tilt of Tyr326-phenol ring at the angle of about 15 de-

gree. This small tilt was induced by the binding of Tyr326-phenol ring to the phenol A ring of 4-α-cumylphenol (Fig. 4A). The Val313-isopropyl group is in the tight interaction with the Tyr326-phenol phenyl group in apo-ERRγ-LBD (Fig. 4C(b)).

All these results imply that the binding of chemicals, particularly BPA to ERRγ-LBD, is just an induced-fit-type binding at the Glu275, Leu309, Tyr326, and Leu345 residues. Due to these conformation changes, BPA and 4-α-cumylphenol are able to bind to ERRγ-LBD in a space-filling manner. Such tolerable flexibility of the ligand-binding pocket must be to adopt an authentic ligand probably with a BPA-like structure.

4-α-Cumylphenol as a phenol ligand of ERRγ in an activation conformation

Both H12 in the BPA/ERRγ-LBD complex and H12 in apo-ERRγ-LBD are in the transcriptionally active conformation [8]. H12 is placed rigidly on the LBD body, where the coactivator binds. ERRγ *per se* elicits a very high basal activity in the luciferase reporter gene assay, and BPA sustains this high spontaneous constitutive activity of ERRγ [6]. These findings were reproduced for the 4-α-cumylphenol/ERRγ-LBD complex, H12 in the complex being in the activation conformation. 4-α-Cumylphenol has also been shown to retain ERRγ's high spontaneous constitutive activity [18].

4-Hydroxytamoxifen (4-OHT) deactivates the ERRγ receptor, dissociating the H12 region from the LBD body [19]. It should be noted that BPA and 4-α-cumylphenol reverse this deactivation activity of 4-OHT, displacing 4-OHT to reposition the H12 from the transcriptionally inactive conformation to the active conformation. The compounds that deactivate the receptor are termed 'inverse agonists,' whereas those that inhibit such inverse agonists are to be defined as 'inverse antagonists.'

In the present study, we found that ERRγ-LBD adopts both BPA and 4-α-cumylphenol, but BPA requires the back-and-forth rotation of the Leu345-isobutyl group. Thus, we should define 4-α-cumylphenol as a genuine space-filler of ERRγ. This implies that the phenol compounds can become a potent ligand of ERRγ. In fact, it was found that a number of phenols bind to ERRγ very strongly [18]. These results suggest that ERRγ may have a phenol-containing endogenous ligand.

It is now important to examine whether or not the phenol compounds including 4-α-cumylphenol cause low-dose effects similar to those reported for BPA. At the same time, it is necessary to clarify what the physiological roles of ERRγ are, and to examine the extent of, and direction in which, BPA and phenols may influence these. This is particularly important because ERRγ is expressed very strongly in the mammalian fetal brain and also in the placenta, at sites that could have important outcomes for newborns.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.06.050.

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The Japanese Peptide Society (2009)

Bisphenol A-Specific Nuclear Receptor ERRγ: Structure-Function Analysis of the Two Novel Isoforms Lacking Vital Peptide Fragment in the Ligand Binding Domain

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We have demonstrated that ERRγ binds strongly bisphenol A (BPA), one of the nastiest endocrine disruptors, retaining ERRγ's high basal constitutive activity. In the present study, we found the two different LBD-wrecked ERRγ isoforms, which are abundant (about 20%) broadly in various human tissues. These ERRγ isoforms were suggested to affect the sound ERRγ as a regulatory element.

Keywords: bisphenol A, endocrine disruptor, ERRγ, isoforms, nuclear receptor

Introduction

Estrogen-related receptor γ (ERRγ), one of 48 human nuclear receptors, is in a fully activated conformation with no ligand. We have recently demonstrated that ERRγ binds strongly bisphenol A (BPA) with the high basal constitutive activity [1]. In the *in vivo* animal experiments, the low-dose effects of BPA have been evidenced, and thus the adverse effects of ERRγ are very much doubtful. Although we found that human placenta and brain stem express predominantly one of ERRγ mRNA variants, little is known about the intrinsic molecular mechanism of ERRγ functions. In the present study, we revealed the molecular multiplicity of ERRγ mRNAs and proteins, which must be correlated to the multiplicity of physiological functions.

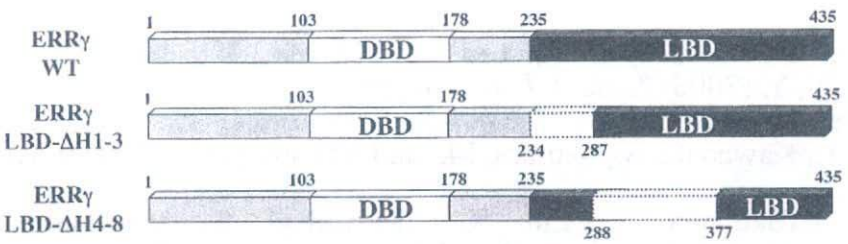

Fig. 1. The three different types of human ERRγ isoforms with wrecked LBD.

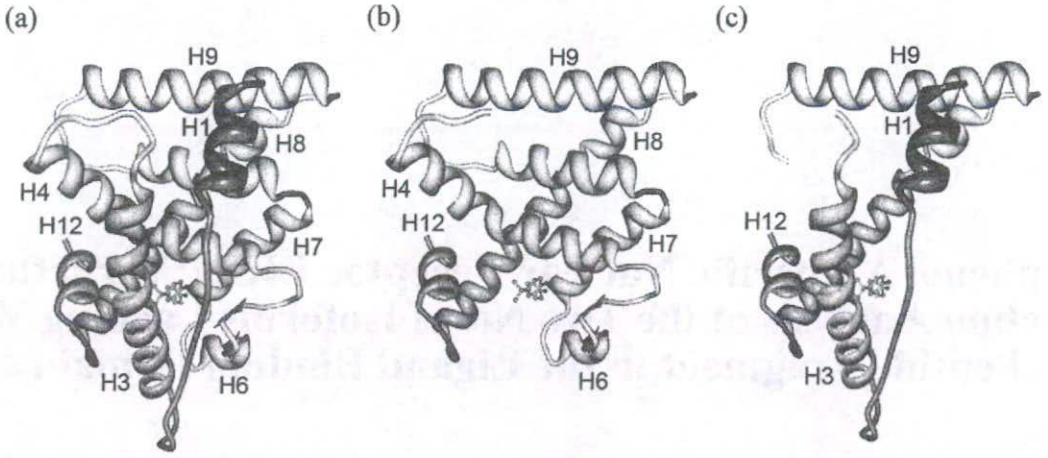

Fig. 2. 3D-Structure of the wild type ERRγ-LBD and its LBD-wrecked forms. (a) ERRγ LBD-WT, (b) ERRγ LBD-ΔH1-3, and (c) ERRγ LBD-ΔH4-8.

Results and Discussion

We eventually identified total 34 mRNA splicing variants from human tissues. As a result, these variants were established to produce 10 protein isoforms. Among these isoforms, two distinct isoforms have a wrecked or broken LBD due to the lack of a peptide fragment either 234-287 or 288-377 (Fig. 1). Those were designated as ERRγ LBD-ΔH1-3 and ERRγ LBD-ΔH4-8, respectively. By means of real-time PCR measurement, these LBD-wrecked isoforms were found to be considerably abundant (3-6% and 12-20%, respectively) broadly in human tissues, suggesting their physiological significance. It should be noted that those do not exhibit any constitute activity in the reporter gene assay.

The LBD of nuclear receptors is constructed by 12 highly conserved α-helix peptides (H1 - H12). Structural evidence for BPA binding to ERRγ-LBD revealed importance of the receptor residues such as Glu275, Arg316, Tyr326, and Asn346 in the ligand-binding pocket [2-4]. The polar amino acids Glu275, Arg316, and Asn346 play central role in forming the hydrogen bonding of two BPA-phenol-OH groups. Surrounding hydrophobic bonds, especially those by Tyr326, reinforce BPA's specific binding. These critical residues are located in H3 (Glu275), H4 (Arg316), and H7 (Asn346), respectively. Tyr326 is in a β-sheet structure located in H4 and H6. As shown in Fig. 2, ERRγ LBD-ΔH1-3 just wrecks H1 and H3, while ERRγ LBD-ΔH4-8 is devoid of the peptide fragment corresponding to H4 - H8. It was strongly suggested that these LBD-broken isoforms cannot bind BPA in their incomplete ligand pockets. Since both isoforms retain H10 essential for functional receptor dimerization, those are probably a regulatory element of sound ERRγ.

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Induced-fit Type Ligand Binding Guided by Free-rotatory Leu Residue Present in the 7th α-Helix Peptide in the Estrogen-related Receptor γ (ERRγ)

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4-α-Cumylphenol, which lacks an OH group from endocrine disruptor bisphenol A (BPA), binds to ERRγ very strongly and specifically. X-ray crystal structural analysis of the 4-α-cumylphenol/ERRγ complex was performed and the structure was compared in detail with the BPA/ERRγ complex. The result clearly indicated that the strong binding of 4-α-cumylphenol is due to the structural flexibility of Leu-side chain at position 345, resulting in a formation of the ligand binding site in an induced-fit manner.

Keywords: bisphenol A, nuclear receptor, X-ray crystal structural analysis

Introduction

Estrogen-related receptor γ (ERRγ) is an orphan nuclear receptor that belongs to the steroid hormone receptor family. This family contains the estrogen receptors α and β, and androgen receptor, considered to be the main targets of endocrine disruptors. Bisphenol A (BPA) has long been recognized as an estrogenic endocrine disruptor, but BPA binds to estrogen receptor very weakly. We have recently discovered that BPA binds to another nuclear receptor named ERRγ very strongly and specifically [1, 2].

Almost all of the nuclear receptors have a unique ligand binding domain, in which the ligand binding pocket is constructed with 12 α-helices (H1-H12) and 2 β-strands in a sheet (S1). These helices play extremely important role to select a specific ligand for the receptor activation. BPA was found to assemble the amino acid residues from H3, H5, H7, H11, H12, and S1 of ERRγ. BPA binds to ERRγ strongly because of the formation of three hydrogen bonds between the phenol-OH groups of BPA and Glu275 from H3, Arg316 from H5, and also Asn346 from H7, respectively. More recently, we found that, in spite of the lack of one of the two BPA-phenol-OH groups, 4-α-cumylphenol exhibits a strong binding ability to ERRγ as well as BPA [2]. In this study, we performed the X-ray crystal structural analysis of 4-α-cumylphenol/ERRγ complex, and compared in detail with the BPA/ERRγ complex.

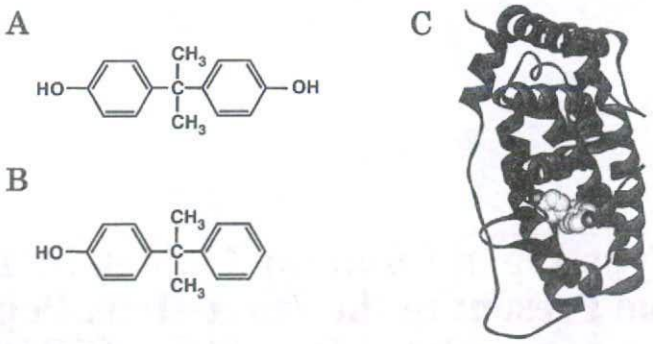

Fig. 1. Chemical structures of bisphenol A (A) and 4-α-cumylphenol(B). Overall structure of 4-α-cumylphenol/ERRγ complex (C).

Results and Discussion

ERRγ was expressed in *E. coli* BL21 as glutathione *S*-transferase (GST) fusion protein. The protein expressed was affinity-purified by using glutathione Sepharose resin, and GST was enzymatically cleaved. The resulting protein was crystallized in the presence of 4-α-cumylphenol. The X-ray crystal structural analysis was performed, and 4-α-cumylphenol/ERRγ complex structure was resolved by molecular replacement method using BPA/ERRγ complex as the searching model. The final model was refined at a 2.0 Å resolution.

The overall structure of 4-α-cumylphenol/ERRγ complex features 12 α-helices (H1-H12) and 2 β-strands in a sheet (S1) without any disordered amino acid residues, and the amino acid residues in the binding pocket were found to be almost the same as those in the BPA/ERRγ complex. Two hydrogen bonds were formed between the remaining OH group of 4-α-cumylphenol and Glu275 or Arg316. Another hydrogen bond of the second BPA-phenol-OH is formed with Asn346 of ERRγ-LBP. Since 4-α-cumylphenol lacks this phenol-OH groups, it should hold substitute residues for a strong receptor binding. When we superimposed the 4-α-cumylphenol/ERRγ-LBD complex with the BPA/ERRγ-LBD complex, we found that the Leu345-isobutyl group rotates 180° completely around the $_{\alpha}$C-$_{\beta}$C bond. ERRγ changes its conformation of Leu345 side chain by the back-and-forth rotation to receive either phenol (BPA) or phenyl (4-α-cumylphenol). Since the Leu conformation in the 4-α-cumylphenol complex was found to be the same as in the apo-form, it become evident that that Leu345 does made a rotation of about 180° to adopt the phenol group of BPA. The crystal structure of the ERRγ complex with (or without) chemicals clearly revealed that ERRγ's Leu345-β-isoprorpyl plays a role in the tight binding of 4-α-cumylphenol and BPA, rotating in a back-and-forth induced-fit manner.

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ERα/ERRα Nuclear Receptor Heterodimer Directly Linked by A Flag Peptide

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It has recently been suggested that, in the tissues where both ERα and ERRα are present together, these nuclear receptors form a heterodimer to function specifically for characteristic physiological role with some efficacy. In the present study, we prepared expression vectors, which produce a recombinant protein of human ERα and ERRα cDNAs cross-linked directly with the FLAG peptide (DYKDDDDK). It was found that ERα in this heterodimer retains the ordinary characteristics of free ERα.

Keywords: estrogen receptor (ER), estrogen-related receptor (ERR), heterodimer, FLAG peptide, nuclear receptor

Introduction

Estrogen receptor α (ERα) and estrogen-related receptor α (ERRα) belong to steroid hormone receptors, or group III of nuclear receptor superfamily. These nuclear receptors function as a transcription factor, binding to their response element present in their target genes and facilitating their transcriptional role in the mRNA biosynthesis. ERRα binds to the ERR-response element (ERRE) as a monomer. ERRα also has an ability to form a homodimer for binding to functional estrogen response elements (EREs) in ER target genes. On the other hand, ERα can bind to ERRE *in vitro* as a dimer. These suggest that the transcriptional activation functions of ERRα and ERα must be overlapped *in vivo* [1].

There are some tissues where both ERα and ERRα are present together. In those tissues, ERα and ERRα have been suggested to form a heterodimer to function specifically for a certain characteristic physiological efficacy. In order to clarify such a dimer-specific effectiveness, it is essential to prepare a concrete heterodimer between ERα and ERRα in the cell nucleus. When they were merely co-expressed, homodimers of each ERα and ERRα would be produced simultaneously. Thus, in the present study, we prepared a gene construct bearing these two receptor proteins successively in a single chain.

Results and Discussion

Since the nuclear receptor protein biosynthesized should be transported from cytoplasm to nucleus to function as a transcription factor, it is necessary to ensure the synthesis of heterodimer and the transcriptional activity of each unit of nuclear

receptors. To this end, we prepared two expression vectors in which human ERα and ERRα cDNAs are cross-linked together via cDNA of FLAG peptide (DYKDDDDK), producing the cDNA conjugates ERα-FLAG-ERRα and ERRα-FLAG-ERα. These vectors were transiently transfected into the HeLa cells, and the expression of dimer protein was confirmed by the Western blotting analysis using anti-FLAG antibody. Intracellular localization of the heterodimers was also ascertained by fluorescence microscopy observation using anti-FLAG antibody. The heterodimers as well as ERα and ERRα were found both in the cytoplasm and nucleus (Fig. 1).

Functional activities of receptors were evaluated by the binding assay for specific ligand interaction and the luciferase reporter gene assay for biological activity. We first examined ERα by using its endogenous agonist ligand 17β-estradiol (E2). In the radio-labeled receptor binding assay, [^{3}H]E2 was found to bind strongly to the heterodimers as in the case of ERα, with the K_D value of about 2 nM (Fig. 2). We also analyzed the transcriptional activity of these heterodimers against ERE or ERRE by reporter gene assays. Transcriptional ability of the heterodimers was found to increase by addition of E2 in a dose-dependent manner. These results indicated that ERα in this heterodimer retains its original characteristics of free ERα. ERα in the heterodimer is probably the influencer of ERRα.

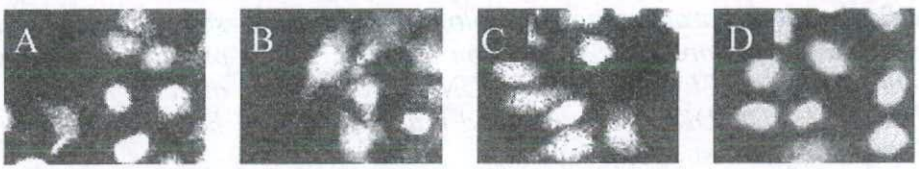

Fig. 1. Intracelluar localization of the nuclear receptor monomers and their heterodimers. ERα (A), ERRα (B), ERα-FLAG-ERRα (C), and ERRα-FLAG-ERα (D). All the receptors were stained by the indirect immunofluorescence method using fluorescein isothiocyanate (FITC)-conjugated secondary antibody with anti-FLAG antibody.

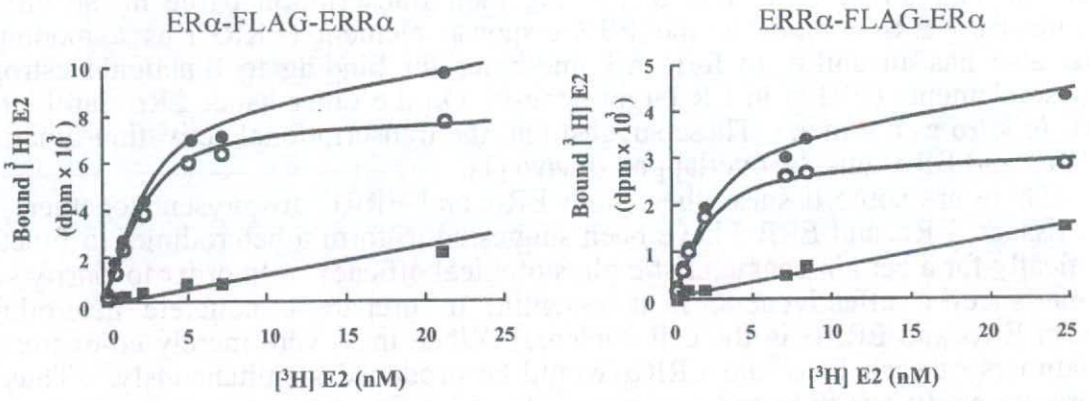

Fig. 2. The results of saturation binding assay for the nuclear receptor heterodimers.

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Placenta Expressing the Greatest Quantity of Bisphenol A Receptor ERRγ among the Human Reproductive Tissues: Predominant Expression of Type-1 ERRγ Isoform

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Estrogen-related receptor γ (ERRγ), one of the 48 human nuclear receptors, has a fully active conformation with no ligand. We recently demonstrated that ERRγ binds strongly bisphenol A (BPA), one of the nastiest endocrine disruptors, and thus retaining ERRγ's high basal constitutive activity. A report that BPA accumulates in the human maternal–fetal placental unit has led us to hypothesize that a large amount of ERRγ might exist in the human placenta. Here we report evidence that placenta indeed expresses ERRγ exceptionally strongly. We first ascertained the presence of nine different ERRγ mRNA variants and the resulting three ERRγ protein isoforms. By real-time PCR, we estimated the relative amount of ERRγ mRNA using total RNA extracts from human reproductive tissues. Placenta was found to express ERRγ extremely highly. Among the three ERRγ protein isoforms, placenta exclusively expresses the type-1 isoform, which possesses additional 23-mer amino-acid residues at the N-terminus of the ordinary ERRγ. This N-terminal elongation was found to elevate by approximately 50% the basal constitutive activity of ERRγ, as evidenced in the luciferase reporter gene assay. The present results suggest that BPA accumulates in the placenta by binding to ERRγ.

Key words: alternative splicing, bisphenol A receptor, estrogen-related receptor γ, placenta, real-time PCR.

Abbreviations: AR, androgen receptor; BPA, bisphenol A; ER, estrogen receptor; ERR, estrogen-related receptor; ERRE, ERR-response element; ERRγ, estrogen-related receptor γ; NRs, nuclear receptors; 4-OHT, 4-hydroxytamoxifen.

INTRODUCTION

Bisphenol A (BPA), 2,2-*bis*(4-hydroxyphenyl)-propane, is one of the highest volume chemicals produced worldwide as a starting material for polycarbonate plastics and epoxy resins. Long known as an estrogenic chemical, BPA is suspected of interacting with human estrogen receptor ER (*1*, *2*) or acting as an antagonist for a human androgen receptor (AR) (*3*, *4*). However, BPA's binding to ER and AR and its hormonal activity are extremely weak: 1,000–10,000 times weaker than with natural hormones.

Based on the idea that BPA may interact with nuclear receptors (NRs) other than ER and AR, we screened a series of nuclear receptors and eventually explored estrogen-related receptor γ (ERRγ) as the BPA target receptor. BPA was found to bind strongly to ERRγ with high constitutive basal activity (*5*–*7*). BPA's binding to ERRγ was further demonstrated by X-ray crystallographic analysis of the complex between BPA and ERRγ (*8*, *9*).

In our efforts to explore the genuine characteristics of ERRγ as a BPA receptor, we have noticed the presence of several different *ERRγ* mRNA isoforms. NRs often possess a number of mRNA isoforms produced by alternative splicing to exhibit functions in a tissue-specific or developmental stage-specific manner (*10*, *11*). However, little is known about the *in vivo* physiological functions of those splicing variants, and even the variants' tissue distributions are poorly understood.

BPA as an endocrine disruptor poses the worrisome threat of low-dose effects on reproductive and developmental processes in humans (*12*). To ensure the presence of *ERRγ* mRNA isoforms in human reproductive organs and brains, we attempted to quantify the total amount of *ERRγ* mRNAs and then the amount of each mRNA isoform. Here we report evidence that the human placenta expresses *ERRγ* mRNA extremely highly, and that the class of isoforms is type-1 ERRγ.

MATERIALS AND METHODS

cDNA Cloning—To confirm the presence of eight reported isoforms of *ERRγ* mRNA, we cloned cDNA by

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using human pancreas and skeletal muscle. These total RNA samples (Clontech, Mountain View, CA, USA) were reverse-transcribed by using the forward primer of ERRγRT1 (5′-GAAAGCTGCTTCATAGTCTTGCTG-3′) and the enzyme SuperScriptII™ RNase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) at 42°C.

To confirm that all clones had inconsistent 5′-UTR sequences, the forward primers were designed separately, depending on the unique structure of each exon (Table 1). As reverse primers, ChERRγR1 and ChERRγR2 were used in the first and the nested PCRs, respectively. As for the amplification of *ERRγ1* cDNA, the first PCR was carried out using a primer set of ChERRγ1F/ChERRγR1 and the enzyme *Pfu Turbo*® Hotstart DNA Polymerase (Stratagene, La Jolla, CA, USA). The second PCR was performed by using PLATINUM® *Taq* DNA polymerase (Invitrogen) with another primer set of ChERRγ1F/ChERRγR2 and the product from the first PCR as a template. For amplification of all other *ERRγ*, PCR was carried out by the same method. Sequence analysis was carried out on CEQ8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA).

Real-Time PCR—The total RNA samples extracted from brains (adult and fetal) and various different reproductive tissues (ovary, uterus, placenta, prostate and testis) were purchased from Clontech, Stratagene and Biochain (Hayward, CA, USA). Each total RNA sample (1 μg) was reverse-transcribed by using SuperScriptII™ (Invitrogen) and oligonucleotide ERRγRT2 (5′-GGAGCAGTCATCATACAG-3′) and hgapdhRT (5′-ATGGTACATGACAA GGTG-3′).

Table 1. **The oligonucleotide sequences of primers used for cDNA cloning of a series of ERRγ mRNA isoform.**

Name of primers	Oligonucleotide sequences
Primers for amplification of ERRγ1 cDNA	
ChERRγ1F	5′-CTGTGCTCTGTCAAGGAAACTTTG-3′
ChERRγR1[a]	5′-GAAAGCTGCTTCATAGTCTTGCTG-3′
ChERRγR2[b]	5′-TTTCAACATGAAGGATGGGAAG-3′
Primers for amplification of ERRγ2 cDNA	
ChERRγ2F	5′-TACGCTAACACTGTCGCAGTTTG-3′
ChERRγ2-adF1	5′-GGTTTTGTAGACTTTCATAGCCAAAG-3′
ChERRγ2-adF2	5′-CGACTCACCTGATTAACCTGCTG-3′
ChERRγR1[a]	5′-GAAAGCTGCTTCATAGTCTTGCTG-3′
ChERRγR2[b]	5′-TTTCAACATGAAGGATGGGAAG-3′
Primers for amplification of ERRγ2-gig cDNA	
ChERRγ2-gigF1	5′-GCCACCACATCTCGATTCAAAG-3′
ChERRγ2-gigF2	5′-CACATGTTCGTGGTGTGGAAAG-3′
ChERRγR1[a]	5′-GAAAGCTGCTTCATAGTCTTGCTG-3′
ChERRγR2[b]	5′-TTTCAACATGAAGGATGGGAAG-3′
Primers for amplification of ERRγ3 cDNA	
ChERRγ3F1	5′-CGGTCCTTCACTTGGAGTTAGTG-3′
ChERRγ3F2	5′-CAAGCTTTATATAGGATCACCGTTGTG-3′
ChERRγR1[a]	5′-GAAAGCTGCTTCATAGTCTTGCTG-3′
ChERRγR2[b]	5′-TTTCAACATGAAGGATGGGAAG-3′
Primers for insertion/deletion confirmation of exon K	
ChERRγJF	5′-CAGAATGTCAAACAAAGATCGACAC-3′
ChERRγLR	5′-CAGCTGAGGGTTCAGGTATGG-3′

[a]The antisense reverse primer R1 has the same nucleotide sequence. [b]The antisense reverse primer R2 has the same nucleotide sequence.

Real-time PCR was performed on a capillary-type LightCycler™ rapid thermal cycler system (Roche Diagnostics, Mannheim, Germany). Reactions were completed in a 10 μl solution mixture and SYBR Green Realtime PCR Master Mix (Toyobo, Tokyo). For normalization, the mRNA gene (*gapdh*) of the enzyme glyceraldehyde-phosphate dehydrogenase was amplified as an internal standard. The assay includes the steps of denaturation at 95°C for 1 min, annealing at 61°C for 3 s and extension at 72°C for a variable time, depending upon the size of products. The product specificity was always confirmed by agarose gel electrophoresis and routinely estimated by the melting curve analysis. To depict the standard curves for quantitative real-time PCR, a 10^{-1}-fold series of dilutions of each plasmid with the same DNA sequence was simultaneously amplified. Quantification of mRNA was achieved using LightCycler software (version 3.5). Standard curves had a correlation coefficient (r^2) of 1.00, linear over a sample concentration range, and mean square error values of 0.03–0.08 were involved.

Western Blotting Analyses—Western blotting was used to detect ERRγ protein isoforms from human kidney and placenta. ERRγ-specific mouse monoclonal antibody was purchased from Perseus Proteomics (Tokyo). The human placenta and kidney lysates were purchased from ProSci (Poway, CA, USA). These lysates (20 μg each) was electrophoresed on 10% polyacrylamide gels. After electrophoresis, gels were electro-blotted onto Hybond-P (GE Healthcare, Waukesha, WI, USA). The blot was incubated overnight in the presence of the anti-ERRγ monoclonal antibody. ERRγ protein was visualized by chemi-luminescence (GE Healthcare) using HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). To discriminate a positive band from negative ones, negative staining controls were performed without the first antibodies.

Reporter Gene Assay for ERRγ Types 1 and 2—Type I and type II ERRγ fragments were cloned into the vector pcDNA3.1(+) (Invitrogen). As an ERR response element (ERRE)-luciferase construct, 3 × ERRR/pGL3 was used as described previously (7). HeLa cells were maintained in Eagle's MEM medium (Nussui, Tokyo) with 10% (v/v) fetal bovine serum at 37°C. HeLa cells were transfected with 3 μg of luciferase reporter gene (pGL3/3 × ERRE), 1 μg of the expression plasmid of the wild-type of either type I or type II ERRγ and 10 ng pSEAP-control plasmid as an internal control by Lipofectamine Plus reagent (15 μl/ml, Invitrogen). Approximately 24 h after transfection, cells were harvested and plated into 96-well plates at a concentration of 5×10^4 cells/well. The cells were then treated with varying doses of chemicals, BPA (Tokyo Kasei Kogyo, Tokyo) and 4-OHT (Sigma-Aldrich, St. Louis, MO, USA), diluted with 1% BSA/PBS (v/v). After 24 h, luciferase activity was measured by using the Luciferase assay reagent (Promega, Madison, WI). SEAP activity was assayed by using Great EscAPe™ SEAP assay reagent (Clontech) according to the Fluorescent SEAP Assay protocol. Light emission was measured on a microplate reader Wallac 1420 ARVOsx (Perkin Elmer, Turku, Finland). Cells treated with 1% BSA/PBS were