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Fine spatial assembly for construction of the phenol-binding pocket to capture bisphenol A in the human nuclear receptor estrogen-related receptor γ

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Various lines of evidence have shown that bisphenol A (BPA) acts as an endocrine disruptor that affects various hormones even at merely physiological levels. We demonstrated recently that BPA binds strongly to human nuclear receptor estrogen-related receptor γ (ERRγ), one of 48 nuclear receptors. Based on X-ray crystal analysis of the ERRγ ligand-binding domain (LBD)/BPA complex, we demonstrated that ERRγ receptor residues, Glu275 and Arg316, function as the intrinsic-binding site of the phenol–hydroxyl group of BPA. If these phenol–hydroxyl↔Glu275 and Arg316 hydrogen bonds anchor the A-benzene ring of BPA, the benzene–phenyl group of BPA would be in a pocket constructed by specific amino acid side chain structures. In the present study, by evaluating the Ala-replaced mutant receptors, we identified such a ligand-binding pocket. Leu268, Leu271, Leu309 and Tyr326, in addition to the previously reported participants Glu275 and Arg316, were found to make a receptacle pocket for the A-ring, whereas Ile279, Ile310 and Val313 were found to assist or structurally support these residues. The results revealed that each amino acid residue is an essential structural element for the strong binding of BPA to ERRγ.

Keywords: bisphenol A/estrogen-related receptor γ/receptor-binding mode/receptor-binding assay.

Abbreviations: BPA, bisphenol A; CBB, Coomassie brilliant blue; DCC, dextran-coated charcoal; DMSO, dimethyl sulfoxide; ERR, estrogen-related-receptor; ERRE, ERR-response element; ERRγ, estrogen-related receptor γ; LBD, ligand-binding domain; LBP, ligand-binding pocket; N_{BPA}, the number of binding site for BPA; ND, not determined; NR, nuclear receptor; NSB, no specific binding.

Estrogen-related receptor γ (ERRγ), one of 48 human nuclear receptors (NRs), is an orphan receptor whose physiological ligand is unknown (*1–3*). We found recently that ERRγ binds strongly to bisphenol A (BPA), which has long been recognized as an estrogenic chemical able of interacting with human estrogen receptor (ER) (*4–8*). The so-called low-dose effects of BPA are continuously being discovered for many organ tissues and systems in mice and rats *in vivo* (*9–13*). However, following the discovery of ERRγ as a BPA-specific receptor, it became crucial to determine whether the previously reported effects of BPA at low doses are mediated through ERRγ and its specific target gene(s) (*14*).

ERRγ *per se* is highly active with no ligand, since ERRγ is constitutively in an activation conformation (*4*, *15–17*). Surprisingly enough, the ERRγ–BPA complex was found to exist in similar activation conformation (*18*). In this complex, a single molecule of BPA stays at the pocket of each ERRγ ligand-binding domain (LBD), and this BPA appears to be a space filler with no detectable influence on the ERR receptor conformation. This strongly suggests that, if ERRγ has an endogenous ligand, it would be quite similar to BPA. Given that there is such an intrinsic ligand, it is important to explore the detailed structural characteristics of the ligand-binding pocket (LBP).

BPA, 2,2-*bis*(4-hydroxyphenyl)propane, has a chemical structure of $HO–C_6H_4–C(CH_3)_2–C_6H_4–OH$ with two phenol groups with A and B benzene rings, and two methyl groups on the sp^3 tetrahedral carbon atom (Fig. 1A). The crystal structure of the complex suggested that a series of amino acid residues are engaged in essential interactions between the BPA and ERRγ-LBD molecules. For example, by examining the ERRγ-LBD analogues through site-directed mutagenesis, we have demonstrated that the phenol–hydroxyl group of the A-ring is anchored by hydrogen bonds with Glu275 (H3) and Arg316 (H5) at one side of the LBP (*19*, *20*). These hydrogen bonds were also observed in the ERRγ–LBD complex with 4-α-cumylphenol, which lacks the phenol–hydroxyl group in the B-ring of BPA (*21*).

For better characterization of the LBP of ERRγ, we carried out a series of site-directed point mutagenesis modifications in the present study. Those amino acids mutated into Ala included all the residues in the proximity (within a distance of 5 Å) of BPA in ERRγ-LBD (Fig. 1B). Mutant receptors were evaluated by the receptor-binding assay using

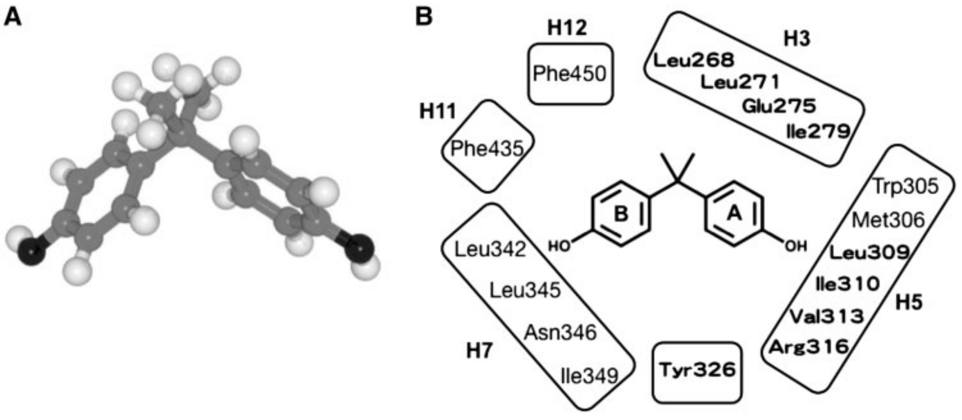

Fig. 1 3D Stereo-structure of BPA and the structural environment of its A-ring in the LBP of the ERRγ. (A) Ball-and-stick model structure of BPA based on the X-ray crystal structure (Protein Data Bank accession code: 2E2R (*18*)). (B) The proximity of each amino acid residue (within a distance of 5 Å) to BPA is shown in the boxes depicting the α-helices. Amino acids shown in a large bold font are the residues close to the benzene A-ring of BPA. This figure was modified from Fig. 4A in Ref. (*18*).

tritium-labelled BPA as a tracer and by the reporter gene assay using HeLa cells. The results indicated that Leu268, Leu271, Leu309 and Tyr326 play an essential role in capturing BPA, and Ile279, Ile310 and Val313 were identified as the residues involved in structurally maintaining these binding sites. We here describe in detail these structural roles of the residues constructing the BPA-binding sites in ERRγ.

Experimental Procedures

Plasmid construction and site-directed mutagenesis

Wild-type ERRγ-LBD encoding 222–458 residues was generated by PCR using the human kidney cDNA library (Clontech Laboratories, Mountain View, CA, USA) and cloned into the vector pGEX-6p-1 (Amersham Biosciences, Piscataway, NJ, USA) or pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) using the EcoRI and XhoI restriction enzyme sites. The resulting plasmids were designated as pGEX-ERRγ-LBD and pcDNA3.1-ERRγ-Full, respectively.

ERRγ mutants were generated using *PfuTurbo*® DNA Polymerase (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions with pGEX-ERRγ-LBD or pcDNA3.1-ERRγ-Full as a template and a series of overlapping sense and antisense primer pairs. The mutations were introduced by PCR mutagenesis in a two-step reaction essentially as reported previously (*19*, *22*). Each mutant LBD or full-length ERRγ was amplified and cloned into the expression vector pGEX-6p-1 or pcDNA3.1(+) at the EcoRI and XhoI sites. All PCR products were checked to confirm the accuracy of their sequences by using a CEQ™ 8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA).

ERRγ-LBD Protein Expression

Glutathione S-transferase (GST)-fused proteins of the wild-type and mutant GST-ERRγ-LBD were expressed in *Escherichia coli* BL21 as described previously (*4*, *19*). The receptor protein was purified using an affinity column of glutathione-Sepharose 4B (GE Healthcare BioSciences Co., Piscataway, NJ, USA). After incubation for 1 h at 4°C, the column was washed three times with PBS containing 0.2% (v/v) Triton X-100 and once with sonication buffer [50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol]. The fusion protein was eluted with 50 mM Tris–HCl (pH 8.0) containing 20 mM reduced glutathione, which was removed by gel filtration on a column of Sephadex G-10 (15 × 100 mm; GE Healthcare) equilibrated with 50 mM Tris–HCl (pH 8.0). The purity was confirmed by SDS–PAGE using 12.5% polyacrylamide gel and stained by Coomassie brilliant blue (CBB). The protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA, USA) (*23*).

Circular dichroism spectra measurements

The proteins of GST-fused and GST-free wild-type ERRγ-LBD and solo GST were measured for their *Circular dichroism* (CD) spectrometry in the 10 mM HEPES solution (pH 7.5) containing 50 mM NaCl, 2 mM $MgCl_2$ and 1 mM EDTA, the concentration (~2 μM) of which was determined by the Bradford method. Spectra were recorded at 25°C on a JASCO J-725 spectropolarimeter (JASCO Co., Tokyo, Japan) in a cell of 0.5 mm path length. Spectra were acquired over the 195–300 nm range at 5 nm/min scan rate. Four scans were accumulated to obtain a mean spectrum, which was normalized by subtracting the buffer scan recorded under the same conditions. The results were eventually analysed by the standard analysis software (JACSO) and expressed the mean residue molar ellipticity [θ].

Since a considerable influence of Cotton effects of GST protein moiety became obvious for the CD spectrum of GST-ERRγ-LBD, GST-free ERRγ-LBD mutants were prepared just for CD measurement from their GST-fused proteins. GST was removed by using a specific enzyme PreScission Protease (GE Healthcare) on an affinity column of glutathione-Sepharose 4B. After applying the solution of GST-ERRγ-LBD mutant protein, the column was left to allow for incubation at 4°C for 4 h, and then eluted with the cleavage buffer 50 mM Tris–HCl (pH 7.0) containing 150 mM NaCl and 1 mM EDTA. The eluate was directly diluted (10–20 times) with 10 mM HEPES buffer to prepare the solution for CD measurement.

Radio-ligand receptor-binding assays

Saturation binding. A saturation binding assay was conducted (*24*) using [^{3}H]BPA (8 Ci/mmol) (Moravek Biochemicals, Brea, CA, USA). The reaction mixture was incubated at 4°C for 2 h with the receptor proteins—GST-fused wild-type ERRγ-LBD or its mutants—in 100 μl of binding buffer [10 mM HEPES (pH 7.5), 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 2 mM CHAPS and 2 mg/ml γ-globulin]. The assay was performed with or without the addition of unlabeled BPA (final concentration of 1.0×10^{-5} M) (Tokyo Kasei Kogyo Co., Ltd, Tokyo, Japan) to quantify the specific and non-specific binding. After incubation with 100 μl of 1% dextran-coated charcoal (DCC) (Sigma-Aldrich Inc., St Louis, MO, USA) (*25*) in PBS (pH 7.4) for 10 min at 4°C, free radioligand was removed by the direct vacuum filtration method using a 96-well filtration plate (Millipore, Bedford, MA, USA) for the B/F separation. The specific binding of [^{3}H]BPA was calculated by subtracting the non-specific binding from the total binding, and the results were examined by Scatchard plot analysis (*26*). The assay was carried out at least three times.

Competitive binding. BPA was dissolved in a binding buffer containing 0.3% dimethyl sulfoxide (DMSO). Competitive binding assays were performed in the presence of GST-fused wild-type ERRγ-LBD or its mutants at the most appropriate concentration of each.

Table I. Genetic codes of point-mutated original and changed amino acid residues in the BPA-binding sites of human NR ERRγ.

Residue number	Original amino acid in ERRγ and its genetic code		Mutated amino acid and its genetic code								
			Ala	Val	Ile	Asp	Gln	Leu	Lys	Phe	His
268	Leu	CTG	GCG	GTG	ATA						
271	Leu	TTG	GCG	GTG	ATA						
275	Glu	GAG	GCG			GAC	CAG	CTG			
279	Ile	ATC	GCC								
309	Leu	TTG	GCG	GTG	ATA						
310	Ile	ATC	GCC								
313	Val	GTC	GCC		ATC			CTC		TTC	
316	Arg	CGG	GCG					CTG	GAG		
326	Tyr	TAT	GCT							TTT	CAT

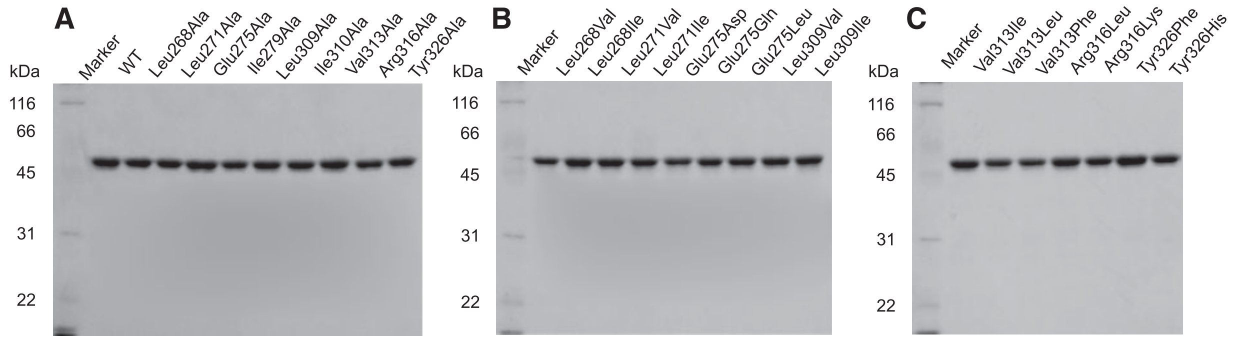

Fig. 2 SDS–PAGE elution profiles of GST-fused LBD of wild-type (WT) ERRγ, GST-ERRγ-LBD and a series of mutants. (A) WT ERRγ and Ala-replaced mutant ERRγ-LBDs, and (B and C) mutant receptors with substitution by amino acid other than Ala. Three micrograms of expressed proteins were separated on 12.5% SDS–PAGE gel and stained by CBB.

Reaction mixtures were incubated with either [^{3}H]BPA (5 nM in final) at 4°C for 2 h, and free radioligand was removed by the method described above after incubation with 100 µl of 1% DCC in PBS (pH 7.4) for 10 min at 4°C. Radioactivity was determined on a liquid scintillation counter (TopCount NXT; Perkin Elmer Life Sciences Japan, Tokyo, Japan). To estimate the binding affinity, the IC_{50} values (the concentrations for the half-maximal inhibition) were calculated from the dose–response curves evaluated by the nonlinear analysis program ALLFIT (*27*). Each assay was performed at least three times.

Cell culture and transient transfection assays

HeLa cells were maintained in Eagle's Modified Eagle Medium (EMEM) (Nissui, Tokyo, Japan) in the presence of 10% (v/v) foetal bovine serum at 37°C under 5% CO_2. HeLa cells were first seeded for 24 h at 5×10^5 cells/dish (6 cm in diameter), and then transfected with luciferase reporter plasmid pGL3/3 × ERRE (3 µg), the expression plasmid of wild-type ERRγ or its mutant (pcDNA3.1(+)/ERRγ-WT or mutant) (1 µg) and 10 ng/dish of pSEAP plasmid as an internal control in the medium (1.0 ml in total) with Plus Reagent (10 µl; Invitrogen) and Lipofectamine LTX (15 µl; Invitrogen). Approximately 24 h after this transfection, the cells were harvested and plated onto 96-well plates at a concentration of 5×10^4 cells per well. The cells were then treated with varying doses of chemicals diluted with 1% BSA/PBS (v/v).

Luciferase activity was measured after 24 h at 37°C under 5% CO_2 by using Luciferase assay reagent (Promega, Madison, WI) according to the manufacturer's instructions. Light emission was measured on a Wallac 1420 ARVOsx microplate reader (Perkin Elmer, Turku, Finland). SEAP activity was assayed by using Great EscAPe^{TM} SEAP assay reagent (Clontech) according to the Fluorescent SEAP Assay protocol (*28*, *29*). Cells treated with 1% BSA/PBS were used as a vehicle control. Values were computed as fold inductions after normalization to SEAP activities. Each assay was performed in duplicate at least three times.

Results and Discussion

Ala-substitutions reveal the structural importance of the BPA-binding site

For the receptor-binding assays, the ERRγ-LBD was expressed in *E. coli* as a protein fused with GST. Site-directed mutations were introduced into a series of amino acid residues, *i.e.* Leu268, Leu271, Glu275, Leu309, Arg316 and Tyr326, by the PCR mutagenesis method (*30*). Their original nucleotide triplet codons were mutated to either GCG, GCT or GCC, which are codons of Ala (Table I). The purity of expressed GST-fused ERRγ-LBD proteins was examined by SDS–PAGE (12.5% polyacrylamide gel). As shown in Fig. 2, all of GST-ERRγ-LBD mutants including the wild-type were judged to be sufficiently pure to use for the receptor-binding assays.

In order to inspect the conformation of ERRγ-LBD mutant receptors, we carried out the measurement of CD spectra of these proteins. For appropriate argument of the structure–activity relationships between the wild-type and the mutant receptors, it is a requisite for the proteins to hold a properly folded conformation without any denaturation/misfolding. Since GST itself is a protein with the structure of mixed α-helices and β-strands (Protein Data Bank accession code: 3GTU) and it was found that GST's Cotton effects affect considerably the CD profile of ERRγ-LBD (Fig. 3A), we decided to prepare GST-free ERRγ-LBD proteins for CD measurement. As shown in Fig. 3B and C, compared with the wild-type ERRγ-LBD

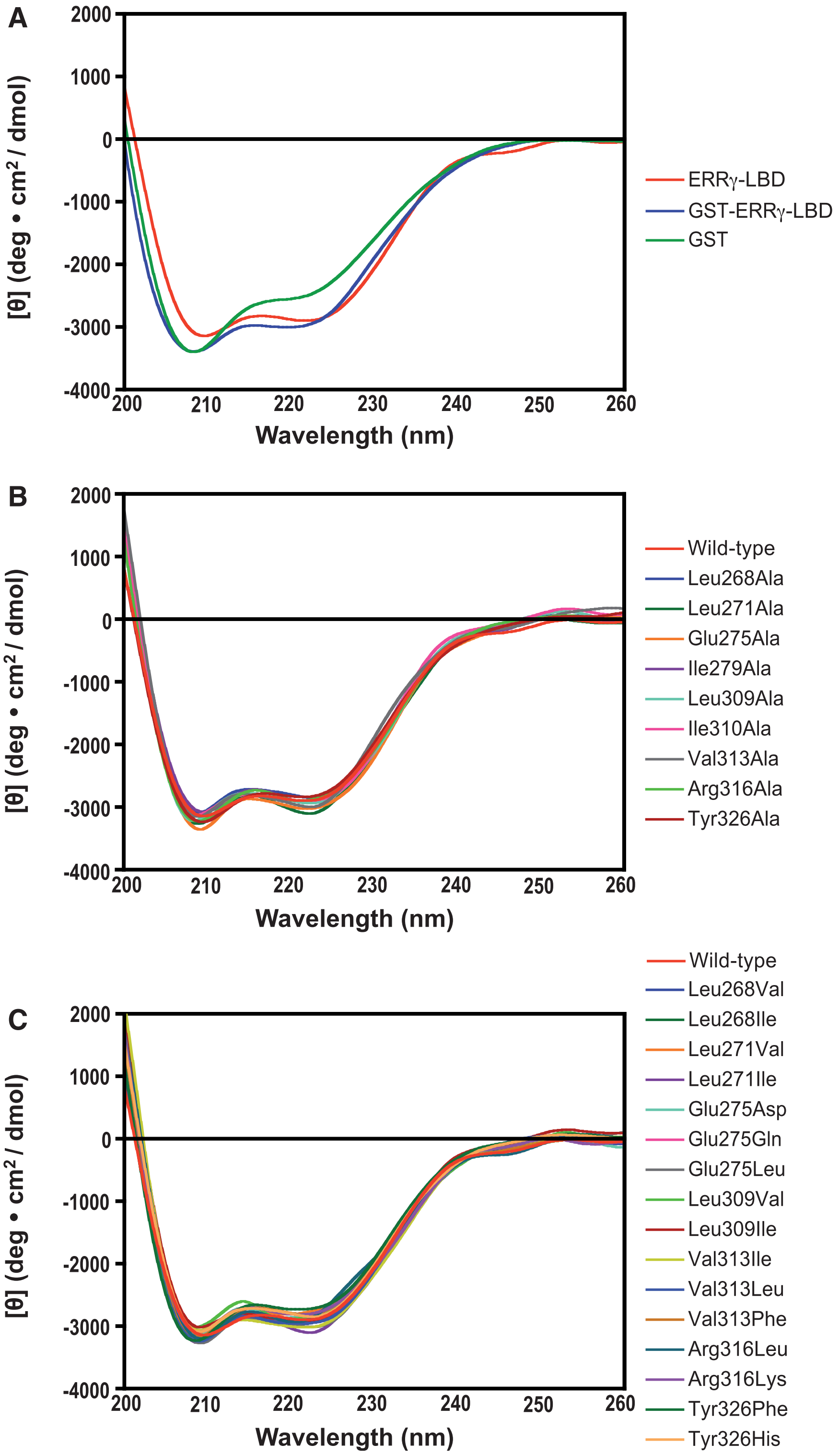

Fig. 3 CD spectra of LBD of WT ERRγ, ERRγ-LBD, and a series of mutants. (A) WT ERRγ-LBD, GST-ERRγ-LBD and solo GST, (B) Ala-substituted mutant ERRγ-LBDs with no GST and (C) mutant receptors with substitution by amino acid other than Ala (ERRγ-LBD with no GST). CD spectra in the far-UV region (200–260 nm) are shown by the mean residue ellipticity [θ] (deg· cm^2/dmol).

protein, all mutant ERRγ-LBD proteins depicted almost the same CD patterns with the two troughs at 208 and 222 nm, respectively. The 208 nm trough was found in a range of ±7.5% deviation as compared with that of the wild-type ERRγ-LBD protein, while the 222 nm trough was in a range of ±7.0% deviation. Thus, the entire mutant ERRγ-LBD proteins appear to be in a well-folded and sound α-domain structure with no denaturation/misfolding.

To evaluate the ligand-binding ability of the mutant receptors, a saturation-binding assay was first performed using GST-ERRγ-LBD and [^{3}H]BPA (Fig. 4A).

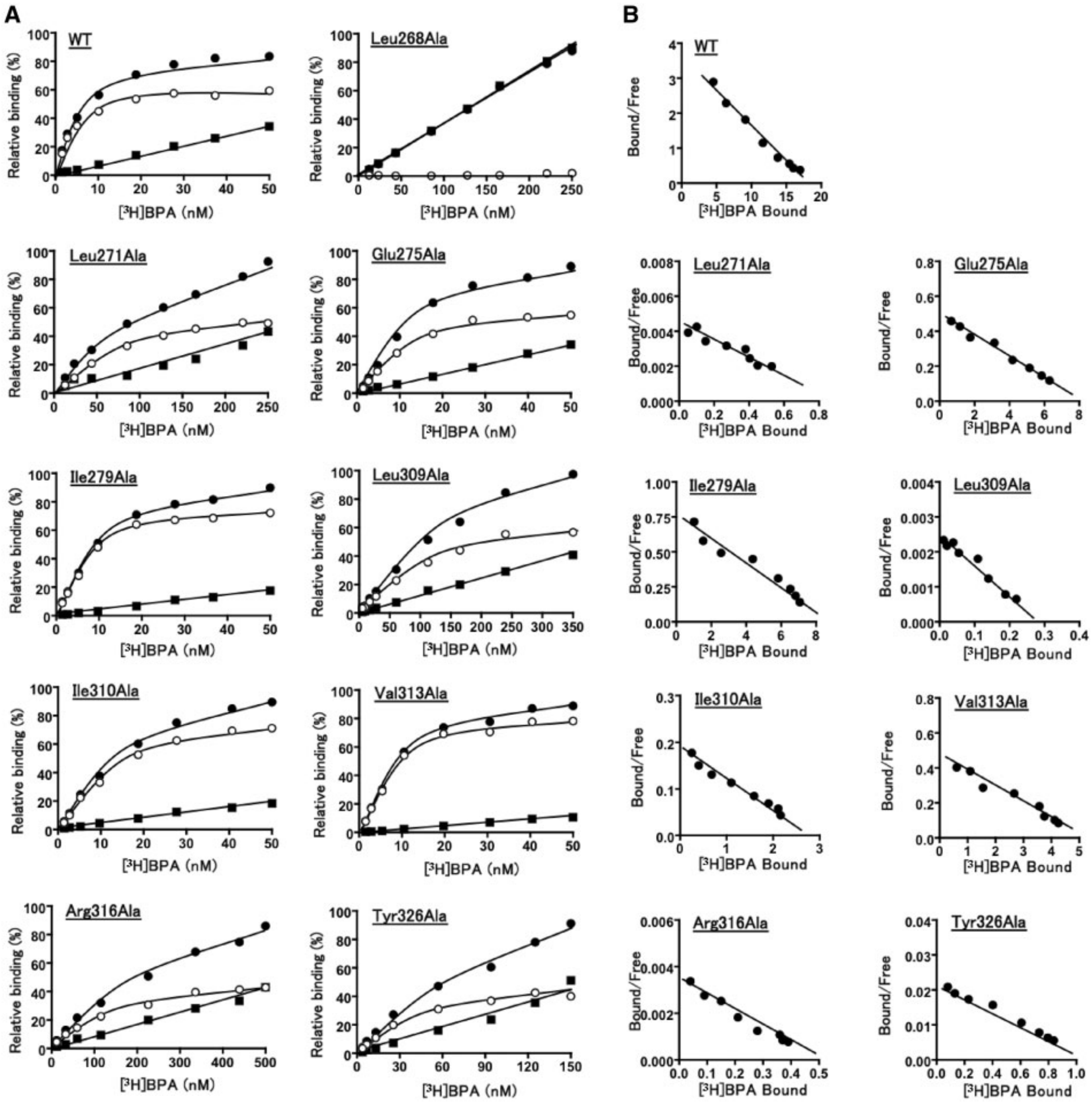

Fig. 4 Receptor-binding assays of tritium-labelled BPA for the Ala-replaced mutant ERRγ LBD. (A) Saturation binding assays with the curves of total binding (filled circle), non-specific binding (filled square) and specific binding (open circle). (B) Scatchard plot analyses showing a single binding mode with a binding affinity constant (K_d) and receptor density (B_{max}). No Scatchard plot analysis was carried out for the Leu268Ala ERRγ mutant receptor because of its lack of specific binding in the saturation binding assay. All the saturation binding assays using [^{3}H]BPA were carried out at least three times and the representative analyses that affords the K_d and B_{max} values closely similar to the means are shown for each mutant receptor.

When no specific binding (NSB) was measurable under the same experimental conditions as used for the wild-type ERRγ receptor, the assay was carried out repeatedly for the specified number of times using varied concentrations of the receptor GST-ERRγ-LBD or radio-ligand [^{3}H]BPA. On the other hand, for the mutant receptors exhibiting a sufficient specific binding, Scatchard plot analysis of saturation binding curves estimated the dissociation constant (K_d) and the receptor density (B_{max}) for [^{3}H]BPA as shown in Fig. 4B. It should be noted that the apparent number of BPA molecule bound to one molecule of EPRγ-LBP and its mutants, namely N_{BPA}, can be estimated by the following equation: $N_{BPA} = B_{max} \times \text{M.W.} \times 10^{-6}$, where M.W. is molecular weight of each mutant receptor exhibiting that particular B_{max} value. N_{BPA} corresponds to a quantity ratio between BPA and ERRγ or each Ala mutant receptor, and we calculated N_{BPA} value for all the mutant receptors (Table II). Theoretically, the value of $N_{BPA} = 1$ should be attained, and indeed the wild-type ERRγ exhibited almost the ideal value 0.968 (Table II). This result suggests that expressed GST-ERRγ-LBD protein is well folded and sound as a receptor, affording almost a 1:1

Table II. The apparent number of BPA molecule bound to one molecule of human NR ERRγ and its mutants.

Amino acid residues of ERRγ receptors				
Position	Mutation	Receptor density for [^{3}H]BPA B_{max} (nmol/mg protein)	Molecular weight calculated	N_{BPA}[a]
Wild-type		18.40 ± 0.78	52,382.47	0.968
Leu268	Ala	NSB[b]	52,340.39	–
	Val	NSB	52,368.44	–
	Ile	NSB	52,382.47	–
Leu271	Ala	0.82 ± 0.15	52,340.39	0.043
	Val	2.71 ± 0.64	52,368.44	0.142
	Ile	2.93 ± 0.13	52,382.47	0.153
Glu275	Ala	8.03 ± 2.66	52,324.43	0.420
	Asp	12.42 ± 0.46	52,368.44	0.649
	Gln	7.81 ± 0.47	52,381.49	0.409
	Leu	NSB	52,366.51	–
Ile279	Ala	8.06 ± 0.42	52,340.39	0.422
Leu309	Ala	0.27 ± 0.011	52,340.39	0.014
	Val	NSB	52,368.44	–
	Ile	NSB	52,382.47	–
Ile310	Ala	2.28 ± 0.13	52,340.39	0.119
Val313	Ala	5.35 ± 0.31	52,354.41	0.280
	Phe	NSB	52,430.51	–
	Leu	14.93 ± 0.44	52,396.67	0.781
	Ile	NSB	52,396.67	–
Arg316	Ala	0.53 ± 0.025	52,297.36	0.028
	Lys	9.98 ± 0.76	52,354.46	0.522
	Leu	NSB	52,339.44	–
Tyr326	Ala	1.06 ± 0.29	52,290.37	0.055
	Phe	18.00 ± 0.89	52,366.47	0.943
	His	6.42 ± 0.24	52,356.44	0.336

All the saturation binding assays to determine the receptor density (B_{max}) for [^{3}H]BPA were carried out at least three times.

[a] N_{BPA} means the apparent number of BPA molecule bound to one molecule of ERRγ-LBP or its mutants and its value was calculated from the values of B_{max} and molecular weight.

[b] NSB means 'no specific binding' in the saturation binding assay.

stoichiometry. However, as shown in Table II, almost all the mutant receptors exhibited values substantially less than 1. It is noteworthy that there is a clear tendency, in which the receptor with a higher K_d value, or a significant decrease of the apparent B_{max} value has a decreased N_{BPA} value. This may be due to competition between the mutants and additives used in the binding-assay system such as γ-globulin or DCC.

Leu268. In this saturation-binding assay, the ERRγ receptor with Ala-substitution at position 268, namely, the Leu268Ala-ERRγ mutant receptor, exhibited NSB (Fig. 4) (Tables II and III), indicating that Leu268 is crucial for the binding of BPA to ERRγ. Elimination of an isopropyl group from the β-methylene of the Leu side chain, resulting in a conversion of Leu into Ala, appeared to destroy the structure of the LBP of ERRγ. [^{3}H]BPA did not exhibit any specific binding even when administered at 30 nM concentration. The Leu268 side chain was thus clearly essential to hold the benzene-A ring of BPA.

Leu271. In addition to the Leu268 residue, the Leu residue at position 271 was found to have critical importance. The absolute necessity of Leu271 is clear, since the Leu271→Ala substitution, namely the elimination of the isopropyl group of the β-methylene of the Leu side chain, resulted in a fatal loss of effective receptor population, with a receptor density $B_{max} = 0.82$ nmol/mg protein, or the number of BPA-binding site $N_{BPA} = 0.043$ (Fig. 4) (Table II). Furthermore, the value of the dissociation constant K_d, which designates the ability of [^{3}H]BPA to bind the receptor, was estimated to be 194 nM, more than 30 times weaker than that for the wild-type ERRγ (Table III). Again, the Leu side chain was clearly essential to hold the benzene-A ring of BPA.

Leu309. A similar importance of the Leu side chain was demonstrated at the position of 309. The Leu309→Ala substitution resulted in a critical loss of activity to bind [^{3}H]BPA ($K_d = 110$ nM), receptor density ($B_{max} = 0.27$ nmol/mg protein) and the number of BPA-binding site ($N_{BPA} = 0.014$) (Tables II and III). Leu309 is essential and critical to retain BPA. Thus, the Leu residues at positions 268, 271 and 309, all of which are within 5 Å of the BPA-A benzene ring, were demonstrated to be necessary to preserve BPA. The interaction of these Leu-isopropyl $(CH_3)_2CH$ groups and the BPA-A benzene ring must be characterized by the hydrophobic interaction, including the so-called CH/π interaction.

Glu275 and Arg316. In the previous study using [^{3}H]BPA, Glu275 and Arg316 in ERRγ were found to be essential for binding of BPA to ERRγ-LBD (*19*, *20*). This was also demonstrated in the present study, as shown by the dissociation constant K_d, the receptor density B_{max} and the number of BPA-binding

Table III. Tritium-labelled BPA-binding characteristics of human NR ERRγ and its mutants, in which the amino acid residues considered to be BPA-binding sites were substituted.

Amino acid residues mutated to Ala	Receptor-binding ability of BPA	
Position	Dissociation constant of $[^3H]$BPA (K_d, nM)	Binding affinity of BPA (IC_{50}, nM) in $[^3H]$BPA displacement assay
Wild-type	5.70 ± 0.88	4.96 ± 0.71
Leu268	NSB[a]	ND[b]
Leu271	194 ± 17.3	1057 ± 155
Glu275	16.6 ± 1.84	30.2 ± 3.09
Leu309	110 ± 14.0	365 ± 69.2
Arg316	168 ± 13.0	622 ± 14.3
Tyr326	47.3 ± 4.8	61.4 ± 9.92

All the binding assays using $[^3H]$BPA were carried out at least three times.

[a] NSB means 'no specific binding' in the saturation binding assay.

[b] Not determined. Because there was NSB in the saturation binding assay, the competitive binding assay could not be carried out.

site in Tables II and III. Although the Ala substitutions of these residues diminished the binding affinity of $[^3H]$BPA to ERRγ, the Arg316→Ala substitution elicited a much more highly reduced affinity (an approximately 30-fold decrement as compared with the affinity of wild-type ERRγ) than the Glu275→Ala substitution (an approximately 3-fold decrease). These results demonstrate that both the Glu275 and Arg316 residues are involved in the hydrogen bonds with the hydroxyl group in the phenol A-ring, but with different degrees of involvement in the hydrogen bonding. In the present detailed investigation using higher concentrations of $[^3H]$BPA and receptor protein, the results clearly indicated that the residual importance of Arg316 is much more significant and crucial, since Arg316Ala was clearly less effective at binding $[^3H]$BPA than expected.

Tyr326. Tyr326 was also assumed to interact with the A-ring of BPA, and thus we attempted to examine whether or not this interaction holds BPA in the LBP of ERRγ. When the Tyr326→Ala substitution was accomplished, the resulting mutant receptor Tyr326Ala-ERRγ was found to provide a significantly reduced specific binding for $[^3H]$BPA (Fig. 4A). The dissociation constant K_d was estimated to be 47.3 nM (Table III), approximately eight times larger than that of wild-type ERRγ, indicating that Tyr326 is essential to retain BPA. The reduced receptor density (B_{max}) and the number of binding site for BPA (N_{BPA}) also indicated that the Tyr-phenol side chain structure is crucial for ERRγ to bind BPA.

The relative importance of the residues for retention of the benzene A-Ring of BPA

It should be noted that all the amino acid residues within 5 Å of the A-benzene ring of BPA increased the value of the dissociation constant K_d of $[^3H]$BPA by their substitution to Ala (Table III). The extent of this increment represents the importance of each amino acid residue for retention of the benzene ring, since an increase in the dissociation constant means a reduction in the binding affinity of $[^3H]$BPA. Accordingly, a comparison of the values of the dissociation constants of each mutant receptor can reveal the relative importance of the residues. The larger the K_d value is, the more important its original residue is. As a result, the relative importance of ERRγ-LBP for BPA was judged to be Leu268 > Leu271 > Arg316 > Leu309 > Tyr326 > Glu275 (Table III).

Identical results were obtained from the competitive binding assays using $[^3H]$BPA and non-tritium labelled BPA. No binding assay was carried out for the Leu268Ala-ERRγ mutant receptor, since this mutant receptor did not exhibit any specific binding of $[^3H]$BPA (Fig. 4). As for the other mutant receptors, the binding activity of BPA was evaluated by the IC_{50} value estimated in displacing $[^3H]$BPA. These values were 1,057, 622, 365, 61.4 and 30.2 nM for Leu271Ala-ERRγ, Arg316Ala-ERRγ, Leu309Ala-ERRγ, Tyr326Ala-ERRγ and Glu275Ala-ERRγ, respectively (Table III). The larger the IC_{50} value, the more important its original residue is. Thus, based on the IC_{50} values, the relative importance of ERRγ-LBP for BPA was judged to be as follows: Leu268 > Leu271 > Arg316 > Leu309 > Tyr326 > Glu275.

The competitive binding assay performed in the present study is termed *homologous*, since the same compound BPA was used as the hot and cold ligand. Homologous competitive binding assays have the same goals as a saturation binding assay. Under the condition that the hot and cold ligands have identical affinities, theoretically, we may observe a linear relationship between the K_d and the IC_{50}. As shown in Fig. 5, it is clear that all the determinations for the mutant receptors together with the wild-type ERRγ are in excellent agreement, as they should be. The relative importance of ERRγ-LBP for BPA binding is thus determined to be in the order of Leu271 > Arg316 > Leu309 > Tyr326 > Glu275. Here, Leu268 should be considered the most important because of its absolute necessity for the BPA binding.

Structural poisoning of the binding sites for benzene A-Ring of BPA

When the X-ray crystal structure of the BPA/ERRγ-LBD complex was displayed by the CPK model, there was a distinct cavity on the surface, and this feature affords a direct view of the hydroxyl–oxygen atom of the benzene A-ring of BPA (Fig. 6F). With this angle fixed, all of the amino acid residues, *i.e.* Leu268, Leu271, Arg316, Leu309, Tyr326 and Glu275, appeared in the right place when the CPK model was converted into the Ribbon model showing only the side chain of residues within 5 Å distance from the benzene A-ring of BPA. In that direction, we could clearly recognize two amino acids, namely Leu309 and Tyr326 (Fig. 6A). At the upper side of the benzene A-ring, Leu309 was located within a distance of 4.29–4.60 Å between the Leu-isobutyl carbon and the BPA-benzene carbon atoms. As shown in Fig. 6B, Leu-isobutyl and the benzene A-ring of BPA participate in the so-called CH/π interaction. At the opposite side, the C2–C3 edge of Tyr326-phenol ring is in close proximity (3.75 Å) to the benzene A-ring of BPA (Fig. 6C). In this conformation, they are in the 'edge-to-face' π–π interaction, although the edge of Tyr326 does not direct straightforwardly to the π-face centre of A-ring. This might be the reason why the relative importance is Leu309 > Tyr326.

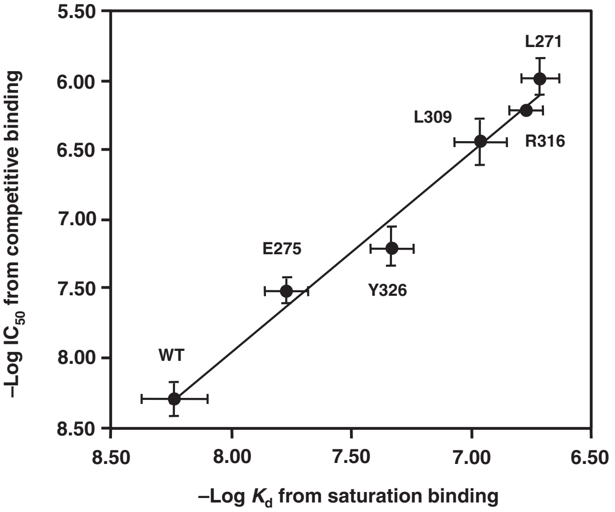

Fig. 5 Interrelationship between the dissociation constant K_d of [^{3}H]BPA in the saturation binding assay and the half maximal IC_{50} of BPA in the homologous competitive binding assay. The plots are shown with the relevant SE, an approximate measure of the 95% confidence limits.

With rotation approximately 90° right anteriorly from Fig. 6A, we catch sight of Arg316 and Glu275 (Fig. 6D). Arg316 was located slightly at the right lower side of the benzene A-ring of BPA, while Glu275 was located at the left upper side. Arg316 and Glu275 were necessary for holding BPA in ERRγ, but with different degrees of involvement in the hydrogen bonding to the phenol–hydroxyl group on the A-ring of BPA. Based on the present finding that the relative importance assumed an order of Arg316 > [Leu309 > Tyr326] > Glu275, it can be concluded that phenol–hydroxyl↔Arg316 hydrogen bonding plays a primary role, while the role of phenol–hydroxyl↔Glu275 hydrogen bonding is only supportive. There must be a distinct direct hydrogen bonding between the phenol–hydroxyl and the Arg316-guanidino group. On the other hand, the Glu275-carboxyl group was found to bind to the phenol–hydroxyl group of BPA indirectly via a water

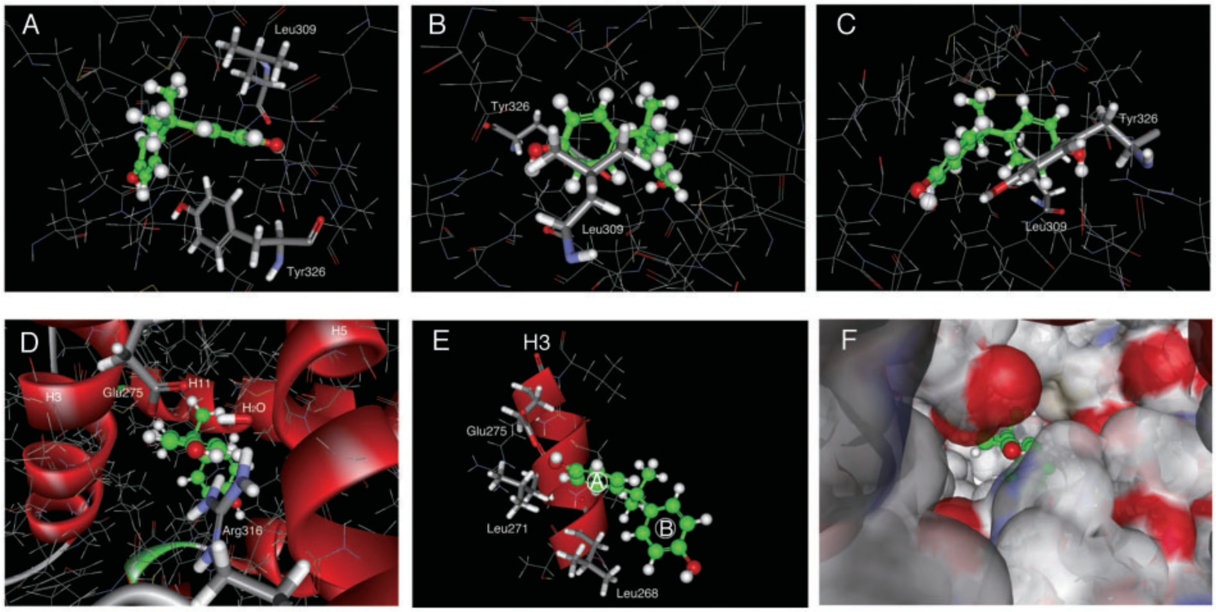

Fig. 6 Characteristic 3D structural views of the BPA-binding sites in ERRγ-LBD. (A–C) A sandwich-type interaction of the benzene A-ring of BPA between the Leu309 and the Tyr326 residues in the BPA-binding pocket. (A) Side view of the interaction, (B) top view from Leu309 and (C) bottom view from Tyr 326. Both γ-methyl groups of Leu309 are in the CH/π interaction with the benzene A-ring of BPA, while the (2–3)-edge of the Tyr326 phenol–benzene ring and the benzene A-ring (π) of BPA are in the 'edge-to-face' π–π interaction. (D) Networks of hydrogen bonds to tether the A-ring phenol–hydroxyl group of BPA. Arg316 and Glu275 themselves are hydrogen-bonding via a water molecule in the BPA-binding pocket. (E) The CH/π interaction between the Leu residues in H3, Leu268 and Leu271, and the benzene A-ring of BPA. Leu268 and Leu271 are adjacent to each other in the same ($i + 3$) ridge of H3, and their isobutyl–methyl groups face the benzene ring. (F) A distinct surface cavity allowing clear visualization of the hydroxyl-oxygen atom of the benzene A-ring of BPA in the BPA-binding pocket. Although there are several cavities that lead into the pocket, there is no gaping hole large enough to allow two-way trafficking of BPA molecules.

Table IV. Tritium-labelled BPA-binding characteristics of human NR ERRγ and its mutants, in which the amino acid residues considered to be BPA-binding sites were substituted.

Amino acid residues of ERRγ receptors		Receptor-binding ability of BPA	
Position	Mutation	Dissociation constant of [^{3}H]BPA (K_d, nM)	Binding affinity of BPA (IC_{50}, nM) in [^{3}H]BPA displacement assay
Wild-type		5.70 ± 0.88	4.96 ± 0.71
Leu268	Val	NSB[a]	ND[b]
	Ile	NSB	ND
Leu271	Val	222 ± 23.3	415 ± 89.8
	Ile	93.2 ± 18.2	203 ± 61.5
Glu275[c]	Asp	22.0 ± 2.86	36.7 ± 7.18
	Gln	23.4 ± 3.34	52.1 ± 8.99
	Leu	NSB	ND
Leu309	Val	NSB	ND
	Ile	NSB	ND
Arg316[c]	Lys	22.5 ± 4.26	37.1 ± 4.73
	Leu	NSB	ND
Tyr326	Phe	8.18 ± 1.41	10.1 ± 0.96
	His	106 ± 7.74	377 ± 33.2

All the binding assays using [^{3}H]BPA were carried out at least three times.

[a] NSB means no specific binding in the saturation binding assay.

[b] Not determined. Since, there was NSB in the saturation binding assay, the competitive binding assay could not be carried out.

[c] The data were taken from studies in the literature (*19*).

molecule (Fig. 6D). It should be noted that the Arg316-guanidino group binds to the same water molecule. This key water molecule is situated at the upper side of the benzene A-ring of BPA in Fig. 6D.

Glu275 is in the α-helix number 3 (H3), and in this H3 there are two other binding spots of BPA, namely Leu268 and Leu271. It should be noted that these two Leu residues are adjacent to each other in the same ($i+3$) ridge of H3, whereas Glu275 and Leu271 are also adjacent to each other in another ridge of ($i+4$). Thus, as shown in Fig. 6E, Leu268, Leu271 and Glu275 are almost in the same surface area of the H3 α-helix, surrounding or encircling the benzene A-ring of BPA.

Replacement of the binding sites for benzene A-Ring of BPA

The amino acid residues of the BPA-binding site were replaced by some other amino acids. Leu268 was replaced by the homologous aliphatic amino acids Val and Ile. However, the resulting mutant receptors were completely unable to bind [^{3}H]BPA, which elicited NSB (Tables II and IV). It is evident that the Val-isopropyl and the Ile-*sec*-butyl side chain groups cannot compensate for the Leu-isobutyl side chain group, indicating the very restricted structural role of Leu268. Exactly the same structural effects were observed for Leu309. Both the Leu309Val and Leu309Ile ERRγ mutant receptors were inactive with NSB of [^{3}H]BPA (Tables II and IV).

In the case of Leu271, the replacements by Val and Ile afforded distinctly altered influences for the binding affinity of BPA. As shown in Table IV, for the Leu271Val ERRγ mutant receptor, BPA exhibited almost the same binding affinity as for the Leu271Ala mutant receptor (Table III). This means that the Val-isopropyl [—$CH(CH_3)_2$] does not compensate for the Leu-isobutyl [—$CH_2CH(CH_3)_2$], probably because of the lack of a methylene CH_2. Instead, the Ile-*sec*-butyl [—$CH(CH_2CH_3)CH_3$] caused the Leu271Ile mutant receptor to partially, but not fully, recover the affinity under the almost same stoichiometry ($N_{BPA} = 0.142$ and 0.153, respectively; Table II). Leu271Ile was still several 10 times less potent than the wild-type ERRγ (Table IV).

As for Glu275Asp, Gln or Leu and Arg316Lys, or Leu ERRγ mutant receptors, exactly the same structure–activity relationships were reproduced as previously reported (Tables II and IV). None of the amino acid replacements for these Glu275 and Arg316 elicited the original binding activity of BPA.

The Tyr326→Ala substitution reduced by 8–12 times the binding affinity of BPA to ERRγ. However, it was found that the Tyr326→Phe replacement, structurally a removal of the *para*-hydroxyl group of Tyr, retains almost the full activity of the wild-type with almost 1:1 stoichiometry ($N_{BPA} = 0.943$) (Table II), indicating that the Tyr side chain-*p*-hydroxyl group is not necessarily important for the binding of BPA. In contrast, the Tyr326→His replacement was found to reduce the ability to bind BPA considerably (20- to 75-fold) (Table IV). This may indicate the importance of the benzene–hydrogen atoms on the Tyr326 side chain phenol group for the CH/π interaction with the benzene A-ring of BPA, as described above.

Amino acid residues as structural essentials for supporting the binding sites of the benzene A-Ring of BPA

It should be noted that the Ala-substitution of Val313 resulted in a considerable reduction in the binding affinities of BPA (two to five times as compared with the wild-type; Table V) and also in the number of binding site for BPA (3.5 times drop in N_{BPA} as compared

Table V. The amino acid substitution effects on the binding affinity of BPA in exploration of the importance of binding site-supporting residues in human NR ERRγ.

Amino acid residues of ERRγ receptors		Receptor-binding ability of BPA	
Adjacent supportive residues[a]	Mutation	Dissociation constant of [^{3}H]BPA (K_d, nM)	Binding affinity of BPA (IC_{50}, nM) in [^{3}H]BPA displacement assay
Wild-type		5.70 ± 0.88	4.96 ± 0.71
Leu268	Ala	NSB[b]	ND[c]
Leu271	Ala	194 ± 17.3	1057 ± 155
Ile279	Ala	11.2 ± 1.73	10.8 ± 0.92
Ile310	Ala	16.5 ± 2.15	21.9 ± 0.99
Val313	Ala	11.0 ± 2.14	26.8 ± 2.12
Val313	Phe	NSB	ND
Val313	Leu	6.59 ± 0.71	16.0 ± 1.41
Val313	Ile	NSB	ND

All the binding assays using [^{3}H]BPA were carried out at least three times.

[a] Adjacent supportive residues correspond to the following supportive BPA binding sites: Leu268 for Leu271; Leu271 for Leu268 and Glu275; Ile279 for Glu275; Ile310 for Leu309; and Val313 for Leu309, Arg316, and Tyr326.

[b] NSB means no specific binding in the saturation binding assay.

[c] Not determined. Because there was no specific binding in the saturation binding assay, the competitive binding assay could not be carried out.

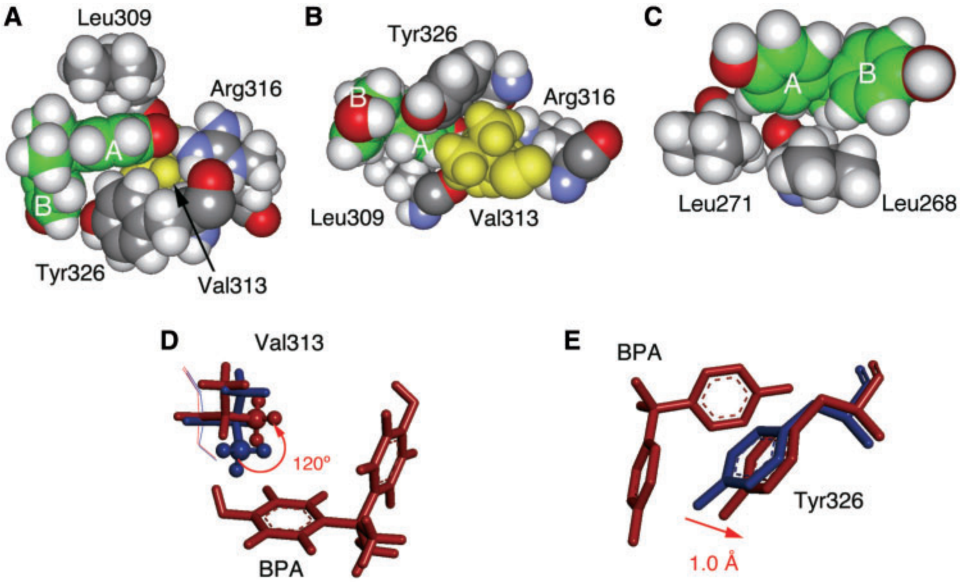

Fig. 7 Structural effects of the Val313 and Leu271 and residues to support the receptor binding of BPA in ERRγ-LBD. (A) Dual structural role of Val313 (yellow) in supporting Arg316 and Tyr326. Arg316 and Tyr326 are the BPA binding site amino acid residues. (B) Intercalation of the benzene A-ring of BPA between Val313 and Leu309, both present in the same ($i + 4$) ridge of α-helix H5. (C) Mutual interrelationship between Leu268/Leu271 and BPA. Leu268 and Leu271 are adjacent to each other in a hydrophobic interaction on the same ($i + 3$) ridge of H3. The Leu268 residue works as a double-hook to bridge both the benzene A- and B-rings of BPA, while Leu271 interacts with the benzene A-ring of BPA and also with Leu268. (D) Rotation (approximately 120°) of the Val313-isopropyl–methyl group that interacts tightly with the Tyr326-phenol phenyl group. Blue stick and ball model shows the Val313-isopropyl group, while the model in red colour shows the Val313-isopropyl group interacting with BPA. (E) Tilt or deviation of Tyr326-phenol ring at the angle of about 15° or with the migration of about 1.0 Å, which was caused by the interaction with BPA. Blue stick model shows the Tyr326 residue, while the model in red shows Tyr326 interacting with BPA.

with the wild-type; Fig. 4B, Table II). In the previous study, it was indicated that the Val313-isopropyl group interacts tightly with the Tyr326-phenol phenyl group in ERRγ-LBD (*21*). This Val313 is also in close proximity to Arg316 (Fig. 7A), because Val313 and Arg316 are adjacent to each other in the same ($i + 3$) ridge of H5. Although Val313 is neighbouring residue of Leu309 in the same ($i + 4$) ridge of H5, the benzene A-ring of BPA intercalates between these residues (Fig. 7B). The influences of this BPA intercalation were observed for Val313 and Tyr326 themselves. For instance, to sustain the tight interaction in the BPA/ERRγ-LBD complex, the isopropyl–methyl group was found to rotate approximately 120°, avoiding a collision with the benzene A-ring of BPA (Fig. 7D). On the other hand, the binding of BPA caused a tilt or deviation of Tyr326-phenol ring at the angle of about 15°, or with the migration of about 1.0 Å (Fig. 7E). Thus, Val313 appears to be a key residue to construct the BPA-binding site in

ERRγ-LBD. As shown in Table V, the Val313→Ala substitution resulted in a distinct drop (two to five times) in the binding activity of BPA. Although the individual contribution of Val313 to the respective adjacent residues cannot be assessed precisely, it is evident that Val313 is crucial for holding the BPA-binding site amino acid residues in the proper positions.

Phe and Ile were found not to replace Val313. As shown in Table V, Val313→Phe or Ile replacement resulted in complete inactivity. In contrast, the Val313→Leu replacement barely sustained the full activity although BPA became slightly weaker (approximately three times) in homologous competition displacement of [^{3}H]BPA (Table V). These results suggested that the major interaction force between Val313 and Leu309, and between Val313 and Arg316, is the so-called hydrophobic bond. Val313 is a linchpin for the successful coordination of a certain number of amino acid residues constructing a BPA-binding site. Similar effects were also observed for some sets of amino acids for other binding sites. These include Ile279 and Ile310 for Glu275 and Leu309, respectively (Fig. 4; Tables IV and V). The Ala-substitution of these residues resulted in a considerable decrease in the binding affinity of BPA (two to four times decrease as compared with the wild-type; Fig. 4 and Table V) and also in the number of binding site for BPA (two to eight times decrease in N_{BPA} as compared with the wild-type; Fig. 4 and Table II). It is also possible to recognize a binding site amino acid as a supporting residue, as seen for Leu268 and Leu271 in the same ($i+3$) ridge of H3 (Table V).

Structural necessity of Leu268 and Leu271 because of additional binding to the benzene B-Ring of BPA

Leu268 and Leu271 are clearly essential to the binding of BPA to ERRγ-LBD. We suspected that these residues would interact with some structural elements of BPA other than the benzene-A ring, and thus we carefully inspected the X-ray BPA/ERRγ-LBD complex structure in detail (*18*). Indeed, it immediately became evident that Leu268 is in close proximity to the benzene B-ring of BPA. Since Leu268 interacts with the benzene A- and B-rings of BPA, this Leu268 is positioned like a clamp or double-hook to tightly connect both of these phenol–benzene rings (Fig. 7C). The shortest distances from the isobutyl–methyl group to these phenol–benzenes were found to be 4.68 and 4.29 Å for the A and B rings, respectively.

Leu271 is in close proximity to the benzene A-ring of BPA, but not to the benzene B-ring of BPA (Fig. 7C). Thus, this Leu residue does not contribute a similar double-hook effect for BPA. However, Leu271 is in close proximity to Leu268, and as a result, Leu271 might function as a double-hook to connect the benzene A-ring of BPA and the Leu268-isobutyl group. This may also result in a strong capturing of BPA molecule in the ERRγ-LBD LBP. It is likely that such a double-hooking clamp effect by Leu271 is inferior to that by Leu268 for BPA itself, and the results from the influence of their Ala-substitution on the receptor activities appear to reflect such a difference in interaction forces.

Effects of Ala-substitutions on the constitutive biological activity

Human NR ERRγ is a so-called self-activating receptor, which is in an activation conformation itself. Thus, ERRγ exhibits almost the full activity with no ligand (*4*). This ERRγ receptor that is capable of producing its biological response in the absence of a bound ligand is said to display 'constitutive activity' (*4*, *17*, *18*). It is intriguing to inspect the effects of Ala-substitution of the binding site amino acids on the constitutive activity. Figure 8 exhibits the results of the luciferase reporter gene assay for all the Ala-substituted ERRγ mutant receptors. It is evident that all the Ala-substitutions influenced and reduced the constitutive activity of the wild-type ERRγ although the rate of reduction varied considerably (50–90%) (Fig. 8). This indicates that the amino acid residues of BPA-binding sites contribute to the construction of a firm activation conformation of ERRγ, and their Ala-substitutions destroy such a sound active conformation. This is also true for the amino acid residues that function supportively for the binding site residues, with the rate of reduction being 30–95%. All the results strongly suggest that the activation conformation of ERRγ is formed or assisted even by the amino acid residues which shape the LBP.

Since BPA exhibited considerably high binding potency for some of Ala-substituted ERRγ-LBD mutant receptors, we expected that a high concentration BPA might rescue the activation conformation damaged by the Ala-substitution. For instance, the binding affinities (IC_{50}) of BPA for Val313Ala, Ile279Ala, Ile310Ala and Glu275Ala were 11.0, 11.2, 16.5 and 16.6 nM, respectively (Tables III and V). Thus, we first examined the effects of BPA for these Ala-substituted mutant receptors in a reporter gene assay with three different BPA concentrations: 0.1, 1 and 10 μM. All of these mutant receptors were indeed rescued to some extent, but the effects were found to be very limited (Fig. 8). This was also true for Leu271Ala, Leu309Ala and Arg316Ala (Fig. 8). These results indicate that the damage by the Ala-substitution is intrinsic for the ERRγ-LBD activation conformation. In particular, the impairment at the Leu309, Ile310, Val313 and Tyr326 residues is irreparable, and thus the importance of these amino acid residues is prominent due to their essential role in the construction or restoration of the activation conformation.

The Leu268Ala mutant ERRγ-LBD receptor did not exhibit any specific binding of [^{3}H]BPA, as mentioned above. In addition, it elicited only ~30% of the constitutive activity of the wild-type ERRγ (Fig. 8). The presence of 0.1 μM of BPA did not affect this low constitutive activity at all. It is evident that Leu268 is essential for construction of the activation conformation of ERRγ-LBD. However, surprisingly, it was found that the activity of the Leu268Ala mutant receptor recovered dramatically upon the addition of increased concentrations of BPA, and at a concentration of 10 μM BPA, this mutant receptor

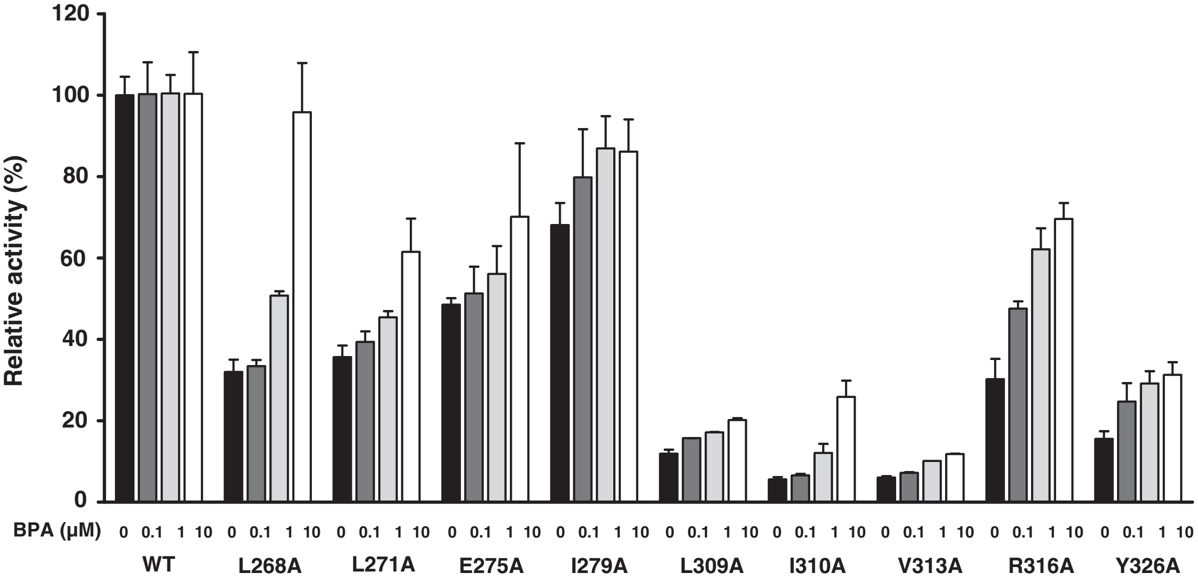

Fig. 8 Luciferase-reporter gene assay of the biological activity of ERRγ and its Ala-substituted mutant derivatives. The percentage relative potencies of a series of mutant receptors were measured against the basal constitutive activity of the WT ERRγ receptor (100%). An internal control that distinguishes the transcriptional level from variations in transfection efficiency was achieved by co-transfecting a second plasmid that constitutively expresses an activity that can be clearly differentiated from SEAP. The assay was carried out in the absence and the presence of BPA to estimate the basal constitutive activity. The BPA concentrations used were 0.1, 1 and 10 μM, and the assays were performed at least three times.

became almost fully active (Fig. 8). This means that BPA can bind to the binding pocket of the Leu268Ala mutant ERRγ receptor when present at an extremely high concentration, and this BPA binding rescues the activation conformation of ERRγ-LBD. These results clearly show that the Leu268 residue is essential for stable or firm binding of BPA, and also for the secure receptor activation conformation.

Conclusion

In the present study, at the BPA benzene A-ring binding site, we carried out an Ala-substitution of amino acid residues in ERRγ-LBD, which were deduced from the X-ray crystal structure analysis of the BPA/ERRγ-LBD complex. Evaluating the receptor binding and biological activities of such Ala-replaced mutant receptors, we confirmed that the residues Leu268, Leu271, Glu275, Leu309, Arg316 and Tyr326 formed a receptacle pocket for the A-ring. In addition, Ile279, Ile310 and Val313 were found to structurally support these residues. Two of the binding site residues, Leu268 and Leu271, were also thought to work as supporting residues. The results revealed that each amino acid residue is an essential structural element for the strong binding of BPA to ERRγ.

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Conflict of interest

None declared.

References

1. Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R.M. (1995) The nuclear receptor superfamily: the second decade. *Cell* **83**, 835–839
2. Robinson-Rechavi, M., Carpentier, A.S., Duffraisse, M., and Laudet, V. (2001) How many nuclear hormone receptors are there in the human genome? *Trends Genet.* **17**, 554–556
3. Horard, B. and Vanacker, J.M. (2003) Estrogen receptor-related receptors: Orphan receptors desperately seeking a ligand. *J. Mol. Endocrinol.* **31**, 349–357
4. Takayanagi, S., Tokunaga, T., Liu, X., Okada, H., Matsushima, A., and Shimohigashi, Y. (2006) Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor gamma (ERRgamma) with high constitutive activity. *Toxicol. Lett.* **167**, 95–105
5. Dodds, E.C. and Lawson, W. (1938) Molecular structure in relation to oestrogenic activity. Compounds without a phenanthrene nucleus. *Proc. Royal. Soc. Lond. B.* **125**, 222–232
6. Krishnan, A.V., Stathis, P., Permuth, S.F., Tokes, L., and Feldman, D. (1993) Bisphenol-A: An estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132**, 2279–2286
7. Olea, N., Pulgar, R., Perez, P., Olea-Serrano, F., Rivas, A., Novillo-Fertrell, A., Pedraza, V., Soto, A.M., and Sonnenschein, C. (1996) Estrogenicity of resin-based composites and sealants used in dentistry. *Environ. Health Perspect.* **104**, 298–305
8. Arase, S., Ishii, K., Igarashi, K., Aisaki, K., Yoshio, Y., Matsushima, A., Shimohigashi, Y., Arima, K., Kanno, J., and Sugimura, Y. (2011) Endocrine disrupter bisphenol A increases in situ estrogen production in the mouse urogenital sinus. *Biol. Reprod.* **84**, 734–742

9. vom Saal, F.S., Cooke, P.S., Buchanan, D.L., Palanza, P., Thayer, K.A., Nagel, S.C., Parmigiani, S., and Welshons, W.V. (1998) A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. *Toxicol. Ind. Health* **14**, 239–260
10. Kubo, K., Arai, O., Omura, M., Watanabe, R., Ogata, R., and Aou, S. (2003) Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats. *Neurosci. Res.* **45**, 345–356
11. vom Saal, F.S. and Hughes, C. (2005) An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. *Environ. Health Perspect.* **113**, 926–933
12. Kamrin, M.A. (2007) The "low dose" hypothesis: Validity and implications for human risk. *Int. J. Toxicol.* **26**, 13–23
13. Kunz, N., Camm, E.J., Somm, E., Lodygensky, G., Darbre, S., Aubert, M.L., Hüppi, P.S., Sisonenko, S.V., and Gruetter, R. (2011) Developmental and metabolic brain alterations in rats exposed to bisphenol A during gestation and lactation. *Int. J. Dev. Neurosci.* **29**, 37–43
14. Okada, H., Tokunaga, T., Liu, X., Matsushima, A., and Shimohigashi, Y. (2008) Direct evidence revealing structural elements essential for the high binding ability of bisphenol A to human estrogen-related receptor γ (ERRγ). *Environ. Health Perspect.* **116**, 32–38
15. Giguere, V. (2002) To ERR in the estrogen pathway. *Trends Endocrinol. Metab.* **13**, 220–225
16. Horard, B. and Vanacker, J.M. (2003) Estrogen receptor-related receptors: orphan receptors desperately seeking a ligand. *J. Mol. Endocrinol.* **31**, 349–357
17. Greschik, H., Wurtz, J.M., Sanglier, S., Bourguet, W., van Dorsselaer, A., Moras, D., and Renaud, J.P. (2002) Structural and functional evidence for ligand-independent transcriptional activation by the estrogen-related receptor 3. *Mol. Cell* **9**, 303–313
18. Matsushima, A., Kakuta, Y., Teramoto, T., Koshiba, T., Liu, X., Okada, H., Tokunaga, T., Kawabata, S., Kimura, M., and Shimohigashi, Y. (2007) Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERRγ. *J. Biochem.* **142**, 517–524
19. Liu, X., Matsushima, A., Okada, H., Tokunaga, T., Isozaki, K., and Shimohigashi, Y. (2007) Receptor binding characteristics of endocrine disruptor bisphenol A for the human nuclear receptor of estrogen-related receptor γ (ERRγ): Chief and corroborative hydrogen bonds of bisphenol A phenol-hydroxyl group with Arg316 and Glu275 residues. *FEBS J.* **274**, 6340–6351
20. Liu, X., Matsushima, A., Okada, H., and Shimohigashi, Y. (2010) Distinction of the binding modes for human nuclear receptor ERRγ between bisphenol A and 4-hydeoxytamoxifen. *J. Biochem.* **148**, 247–254
21. Matsushima, A., Teramoto, T., Okada, H., Liu, X., Tokunaga, T., Kakuta, Y., and Shimohigashi, Y. (2008) ERRγ tethers strongly bisphenol A and 4-α-cumylphenol in an induced-fit manner. *Biochem. Biophys. Res. Commun.* **373**, 408–413
22. Nelson, R.M. and Long, G.L. (1989) A general method of site-specific mutagenesis using a modification of the *Thermus aquaticus* polymerase chain reaction. *Anal. Biochem.* **180**, 47–51
23. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
24. Nakai, M., Tabira, Y., Asai, D., Yakabe, Y., Shimyozu, T., Noguchi, M., Takatsuki, M., and Shimohigashi, Y. (1999) Binding characteristics of dialkyl phthalates for the estrogen receptor. *Biochem. Biophys. Res. Commun.* **254**, 311–314
25. Pichon, M.F. and Milgrom, E. (1973) Competitive protein binding assay of progesterone without chromatography. *Steroids* **21**, 335–346
26. Scatchard, G. (1948) The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* **51**, 660–672
27. DeLean, A., Munson, P.J., and Rodbard, D. (1978) Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.* **235**, E97–E102
28. Berger, J., Hauber, J., Hauber, R., Geiger, R., and Cullen, B.R. (1988) Secreted placental alkaline phosphatase: A powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene* **66**, 1–10
29. Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. 3rd edn, Cold Springs Harbor Laboratory Press, Cold Springs Harbor, NY
30. Heard, D.J., Norby, P.L., Holloway, J., and Vissing, H. (2000) Human ERRγ, a third member of the estrogen receptor-related receptor (ERR) subfamily of orphan nuclear receptors: tissue-specific isoforms are expressed during development and in the adult. *Mol. Endocrinol.* **14**, 382–392