A Characteristic Back Support Structure in the Bisphenol A-Binding Pocket in the Human Nuclear Receptor ERRγ

Xiaohui Liu1, Ayami Matsushima1, Miki Shimohigashi2, Yasuyuki Shimohigashi1*

1 Laboratory of Structure-Function Biochemistry, Department of Chemistry, Faculty of Sciences, and Risk Science Research Center, Kyushu University, Fukuoka, Japan, 2 Division of Biology, Faculty of Science, Fukuoka University, Fukuoka, Japan

Abstract

The endocrine disruptor bisphenol A (BPA) affects various genes and hormones even at merely physiological levels. We recently demonstrated that BPA binds strongly to human nuclear receptor estrogen-related receptor (ERR) γ and that the phenol-A group of BPA is in a recepacle pocket with essential amino acid residues to provide structural support at the backbone. This led BPA to bind to ERRγ in an induced-fit-type binding mode, for example, with a rotated motion of Val313 to support the Tyr326-binding site. A similar binding mechanism appears to occur at the binding site of the BPA phenol-B ring. X-ray crystal analysis of the ERRγ-ligand-binding domain/BPA complex suggested that the ERRγ receptor residues Leu342, Leu345, Asn346, and Ile349 function as intrinsic binding sites of the BPA phenol-B, whereas Leu265, Leu268, Ile310, Val313, Leu324, Tyr330, Lys430, Ala431, and His434 work as structural elements to assist these binding sites. In the present study, by evaluating the mutant receptors replaced by a series of amino acids, we demonstrated that a finely assembled structural network indeed exists around the two adjacent Leu342-Asn346 and Leu345-Ile349 ridges on the same α-helix 7 (H7), constructing a part of the binding pocket structure with back support residues for the BPA phenol-B ring. The results reveal that the double-layer binding sites, namely, the orinigal xidg binding sites and their back support residues, substantiate the strong binding of BPA to ERRγ. When ERRγ-Asn346 was replaced by the corresponding Gly and Tyr in ERRα and ERRβ, respectively, the binding affinity of BPA and even 4-hydroxytamoxifen (4-OHT) is much reduced. Asn346 was found to be one of the residues that make ERRγ to be exclusive to BPA.

Citation: Liu X, Matsushima A, Shimohigashi M, Shimohigashi Y (2014) A Characteristic Back Support Structure in the Bisphenol A-Binding Pocket in the Human Nuclear Receptor ERRγ. PLoS ONE 9(6): e101252. doi:10.1371/journal.pone.0101252

Editor: Jean-Marc Vanacker, Institut de Génomique Fonctionnelle de Lyon, France

Received March 18, 2014; Accepted June 4, 2014; Published June 30, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data are included within the manuscript and supplemental data attached.

Funding: Health and Labour Sciences Research Grants for Research on the Risk of Chemical Substances from the Ministry of Health, Labor and Welfare of Japan to Y.S. Grants-in-aid from the Ministry of Education, Science, Sports, and Culture in Japan to Y.S. (19291912 and 22221005), to A.M. (20710053 and 25701008), and to X.L. (23710080 and 25740024). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: shimo@kyudai.jp

Introduction

Bisphenol A (BPA) binds strongly to estrogen-related receptor γ (ERRγ), one of 48 human nuclear receptors [1,2]. Although ERRγ, as well as its subtypes ERRα and ERRβ, is an orphan receptor whose physiological ligand is unknown even today [3], its transcriptional activity seems crucial, especially during differential gene expression and development [4,5]. Also, it should be noted that one of the major physiological roles of the ERRs appears to control cellular energy metabolism [6].

The causes that ERRγ is rich in both the fetal brain and the placenta and that ERRγ is a probable candidate for involvement in prostatic growth and development seem to have important inferences for newborns [4,7,8]. Thus, the risk of exposure to BPA and its endocrine-disrupting activities has been a cause of concern, especially for fetuses, infants, and children, who are particularly vulnerable to the adverse effects of chemicals [9,10]. So-called low-dose effects of BPA are increasingly being identified for many organ tissues and systems in mice and rats in vivo [11–16], and such effects would be magnified in infants and children. Research into the effects of binding between BPA and ERRγ has inevitably led to a hypothesis that the low dose effects of BPA are mediated through ERRγ and its specific target gene(s) [2,17,18].

ERRγ is constitutively active, exhibiting considerably high basal activity due to an activation conformation of the ligand-binding domain (LBD) with no ligand [1,19–21]. Binding of BPA to ERRγ has almost no effect on the original active conformation [22], and thus almost all of the basal activity is preserved [1,2]. ERRγ is a BPA-specific receptor, but the ERRγ-BPA complex behaves in the same manner as solo ERRγ unaccompanied with BPA. This makes the hypothesis mentioned above very difficult to test. Although BPA-binding has no detectable influence on the ERR receptor conformation, our recent studies have explored, for instance, the fact that the isopropyl-methyl group of Val313 rotates approximately 120° to sustain the tight interaction in the BPA/ERRγ-LBD complex and also to avoid a collision with the benzene A-ring of BPA [23]. Val313 is not a direct binding site of BPA, but a residue which assists or structurally supports the direct binding sites of BPA [24]. These results required us to inspect in detail all the BPA-binding sites of ERRγ.

On the central sp3 tetrahedral carbon atom (C*) of BPA, HO-C6H4-CH=C*H(CH3)2-C6H4-OH, there are two methyl groups and two phenol groups, A and B, respectively (Fig. 1). From the X-ray
crystal structure of the BPA/ERRγ-LBD complex, a set of amino acid residues have been suggested to be in close proximity to the phenol-A group, and their roles as binding sites have been demonstrated by site-directed mutagenesis experiments [24]. Those include Glu275 (H3) and Arg316 (H5) for the phenol-hydroxyl group and Leu268, Leu271, Leu309, and Tyr326 for the phenol-A benzene ring, in addition to Ile279, Ile310, and Val313 for structural support of these residues.

When the amino acid residues of the ligand-binding pocket (LBP) of ERRγ were compared with those of ERRα and ERRβ, it was found that Asn346 is replaced by Gly and Tyr in ERRα and ERRβ, respectively. These replacements are the complete discrepancy seen in LBPs among all the ERR family (ERRα, β, and γ). The substitution of Asn to Gly in ERRα would expand the cavity of LBP and possibly allow much more flexible adaptation of ligands, but would perhaps be unfavourable for a tight binding of the BPA phenol-B group. On the other hand, the Asn→Tyr substitution in ERRβ apparently makes the cavity much narrower, which could prevent the binding of BPA to ERRβ.

In the present study, in order to better understand the characteristics of the binding of the BPA phenol-B ring to ERRγ, we carried out a series of site-directed point mutagenesis modifications. In our previous study, we demonstrated that there is a Leu268 in close proximity to the benzene B-ring of BPA, and this Leu268 is positioned like a clamp or double-hook to tightly connect the phenol-A and -B rings of BPA. In the present study, we first carefully checked the X-ray crystal structure of the ERRγ-LBD/BPA complex, including this Leu268, and especially with respect to the regions surrounding the BPA phenol-B ring. It was suggested that the Leu342, Leu345, Asn346, and Ile349 of ERRγ function as essential binding sites of the phenol-B group of BPA. It should be noted that direct binding site receptor residues are present on the same α-helix 7 (H7) of ERRγ-LBD (Fig. 1), forming the distinct adjacent ridges of Leu342-Asn346 and Leu345-Ile349. On the other hand, it was suggested that Leu265, Leu268, Ile310, Val313, Leu324, Tyr330, Lys430, Ala431 and His434 work as structural elements to assist these binding sites. In order to prove this suggestion, we evaluated a series of site-directed mutant receptors, and we carried out receptor-binding assays for expressed ERRγ-LBD mutant receptor proteins by using tritium-labelled BPA. In addition, the transcription activity of full-length mutant receptors was evaluated by a reporter gene assay using the HeLa cell line. The results indicated that Leu342, Leu345, Asn346, and Ile349 indeed play essential roles in capturing a single BPA molecule, and that the supportive roles of other surrounding residues are essential for this BPA binding. We here describe in detail the structure-activity studies on these amino acid residues that form imperative BPA-binding sites in ERRγ.

Materials and Methods

Chemicals

BPA and 4-α-cumylphenol were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). 4-hydroxytamoxifen (4-OHT) was obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). [3H]BPA (8 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA).

Plasmid construction and site-directed mutagenesis

As previously reported [24], wild-type ERRγ-LBD encoding 222-458 residues was generated by PCR using a human kidney cDNA library (Clontech Laboratories, Mountain View, CA, USA) and cloned into the vector pGEX-6p-1 (GE Healthcare Life Sciences, Piscataway, NJ, USA) using the EcoRI and XhoI restriction enzyme sites. Full-length ERRγ was cloned into the vector pcDNA3.1(+)(Invitrogen, Carlsbad, CA, USA). The resulting plasmids were designated as pGEX-ERRγ-LBD and pcDNA3.1-ERRγ-Full, respectively.

A series of ERRγ mutants were prepared according to the manufacturer’s instructions by using FastT Turbo DNA Polymerase (Stratagene, La Jolla, CA, USA) with pGEX-ERRγ-LBD or pcDNA3.1-ERRγ-Full as a template and a set of overlapping sense and antisense primer pairs. The mutations were introduced by PCR mutagenesis in a two-step reaction essentially as reported previously [24,25]. 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. The accuracy of all PCR product sequences was confirmed by using a CEQ™ 8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA).

ERRγ-LBD protein expression

Wild-type and mutant ERRγ-LBD proteins were expressed as glutathione S-transferase (GST)-fused preparations in E. coli BL21 as described previously [1,24,25]. The receptor protein was purified first by affinity chromatography using a column (10×100 mm) of Glutathione-Sepharose 4B (GE Healthcare). After loading the protein solution, the column was incubated for 1 hour to allow the protein to bind to the enzyme. The column was then washed with two column volumes of 0.1 M sodium phosphate buffer (pH 7.5) containing 300 mM NaCl and 10 mM glutathione. The column was then eluted with 10 mL of 0.1 M sodium phosphate buffer (pH 7.5) containing 100 mM NaCl and 25 mM reduced glutathione. The eluted protein was dialyzed against 0.1 M sodium phosphate buffer (pH 7.5) containing 2 mM glutathione, 10% glycerol, and 0.05% sodium azide. The dialyzed protein was concentrated using an Amicon concentrator with a 30K molecular weight cutoff and analyzed by SDS-PAGE and Western blotting.

Figure 1. Characteristic 3D structural views of the bisphenol A-binding site in α-helix 7 (H7) of the ERRγ ligand-binding domain. (A) Side view of the interaction, and (B) top view of the interaction between bisphenol A and amino acid residues. (Protein Data Bank with accession code: 2E2R [22]. doi:10.1371/journal.pone.0101252.g001)
Circular dichroism (CD) spectra measurements

The GST-free ERRγ-LBD proteins were prepared from their GST-fused proteins for the purpose of CD spectra measurement only. GST was removed by using a specific enzyme PreScission Protease (GE Healthcare) on an affinity column of Glutathione-Sepharose 4B. After loading the solution of GST-ERRγ-LBD protein on the column, the resin was left to allow for incubation at 4°C for 4 h, and then eluted with a cleavage buffer of 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol. The eluate was diluted with the same buffer to obtain the solution of approximately 0.2 μM ERRγ-LBD protein for CD measurements. The concentration was determined by the Bradford method [26]. The Tris-HCl buffer used was judged relevant for CD measurements of proteins.

CD spectra were recorded at 5–95°C with 10°C intervals on a JASCO J-725 spectropolarimeter (Jasco Co., Tokyo, Japan) in a cell of 1 mm path length. Spectra were acquired over the 195–300 nm range at a scan rate of 5 nm/min. Four scans were accumulated to obtain a mean spectrum. The buffer solution itself conducted[27] using [3H]BPA. 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 MgCl₂, 1 mM EDTA, 2 mM CHAPS, and 2 mg/ml γ-globulin]. The assay was performed with or without the addition of unlabelled BPA (final concentration of 1.0×10⁻⁵ M) to quantify the specific and nonspecific binding. After incubation with 100 μl of 1% dextran-coated charcoal (DCC) (Sigma-Aldrich) [28] in PBS (pH 7.4) for 10 min at 4°C, the DCC-absorbed free radio-ligand was removed by the direct vacuum filtration method using a 96-well filtration plate (MultiScreen HTS HV, 0.45 μm pore size; Millipore, Billerica, MA, USA) for the bound/free separation. The data on the specific binding of [3H]BPA were first assessed by Scatchard plot analysis [29]. Then, these data were applied to a one-site binding hyperbola nonlinear regression analysis by the software package Prism (GraphPad Software Inc., La Jolla, CA, USA) to measure changes in the receptor density Bₘₐₓ and equilibrium dissociation constant Kₐₜ. The saturation binding assay was performed at least three times.

Competitive binding. BPA was dissolved in a binding buffer containing 0.1% 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 receptor. The reaction mixtures were incubated with [3H]BPA (final concentration 5 nM) at 4°C for 2 h, and the bound/free separation was carried out by the DCC method as described above. Radioactivity was determined on a liquid scintillation counter ‘TopCount NXT’ (PerkinElmer Life Sciences Japan Co., Ltd., Tokyo, Japan). To estimate the binding affinity, the IC₅₀ values (the concentrations for the half-maximal inhibition) were calculated from the dose-response curves evaluated by the nonlinear analysis program ALLFIT [30]. 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% fetal bovine serum at 37°C under 5% CO₂. HeLa cells were first seeded for 24 h at 5×10⁵ cells/dish (6 cm in diameter), and then transfected with luciferase reporter plasmid pGL3/3×ERRc (3 μg), the expression plasmid of pCDNA3.1-ERRγ-Full (wild-type or mutant) (2 μg), and 10 ng/dish of pSEAP plasmid as an internal control in the medium (5 ml in total) with Plus reagent (10 μl; Invitrogen) and Lipofectamine LTX (15 μl; Invitrogen) according to the manufacturer’s protocol. Approximately 24 h after this transfection, cells were harvested and plated onto 96-well plates at a concentration of 5×10³ cells per well.

Luciferase activity was measured after 24 h at 37°C under 5% CO₂ by using a Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Light emission was measured on a Wallac 1420 ARVOx microplate reader (PerkinElmer). SEAP activity was assayed by using the Great EscAPe™ SEAP assay reagent (Clontech) according to the Fluorescent SEAP assay protocol [31,32]. Secreted embryonic alkaline phosphatase (SEAP) is a reporter widely used to study promoter activity or gene expression. Cells treated with 1% BSA/PBS were used as a vehicle control. Values were computed as the fold inductions after normalization to SEAP activities. Each assay was performed in triplicate at least three times.

Statistical analysis

Data are presented as the mean ± SD for indicated number of separate experiments. Statistical difference was determined by two-sided Student’s t test. P-values less than 0.001 were considered significant.

Computational structural analysis of ERRγ/bisphenol A complex

All molecular modeling studies were carried out using the molecular modeling software Discovery Studio 2.5 (Accelrys; San Diego, CA, USA). The protein structure of the ERRγ-LBD/BPA complex was downloaded from the RCSB Protein Data Bank (PDB) (http://www.pdb.org/pdb/home/home.do) (PDB accession code: 2E2R) and hydrogen atoms were added correctly. ERRγ-LBD/BPA complex was visualized and analyzed on the software. To identify amino acid residues constructed the LBP of ERRγ-LBD, the residues were carefully checked for their spatial interrelationships with BPA molecule, especially noting the interatomic distance and angle.

Results and Discussion

Essential amino acid residues as structural elements specific for BPA-binding

In order to successfully analyse the structure-activity relationship in proteins by replacing a series of amino acid residues with...
other amino acids, it is necessary to guarantee the purity of the protein expressed. In the present study, site-directed mutations were introduced into a series of amino acid residues on α-helix No. 7 (H7) of ERRγ. These included Leu342, Leu345, Asn346, and Ile349. Their back support residues such as Leu263, Leu324, Tyr330, Lys330, and His334 were also mutated. The mutations were carried out by the PCR mutagenesis method [24,25]. For receptor-binding assays, mutant receptors were expressed as GST-fused ligand-binding domain (LBD) proteins in E. coli and purified by affinity-chromatography followed by gel-filtration. The purity of GST-fused ERRγ-LBD was examined by SDS-PAGE (12.5% polyacrylamide gel), and all of the GST-ERRγ-LBD mutants including the wild-type were judged to be sufficiently pure for use in the receptor-binding assays (Fig. S1). The purity of GST-free ERRγ-LBD was also guaranteed by SDS-PAGE (Fig. S1).

Another structural inspection that can be used to guarantee the quality of expressed proteins is analysis of the conformational uniformity and stability of ERRγ-LBD mutant receptors. This is particularly important for adequate evaluation and discussion of the structure-activity relationships between the wild-type and the mutant receptor proteins. In this study, the conformational quality of the expressed proteins was verified by the measurement of CD spectra. Because of the considerably large Cotton effects of GST, a protein with a mixed structure of α-helices and β-strands (Protein Data Bank accession code 2GSR) [33], we prepared GST-free ERRγ-LBD proteins for the purpose of CD spectra measurement only, as reported in a previous study [24]. Measuring the temperature-dependent CD spectra (5°C–95°C at 10°C intervals), it was demonstrated that all the proteins that were mutated at the LBD did indeed hold a properly folded conformation without any denaturation/misfolding at the temperature region 5°C–35°C (Fig. S2), which is the standard condition for temperature in the ordinary regular receptor binding assays. The thermal unfolding curves showed the approximately 55°C of the structural melting point (Tm) for all of GST-free ERRγ-LBD proteins (Fig. S2).

ERRγ position 346 requires Asn, but not Gly and Tyr, for specific BPA-binding

ERRγ possesses Gly at position 402, which corresponds to Asn346 in ERRγ, and thus we replaced ERRγ-Asn346 by Gly. Although the resulting Asn346Gly-ERRγ mutant receptor exhibited sufficient specific binding (ca. 70% of the total binding) for [1H]BPA (Fig. S3), the equilibrium dissociation constant $K_d$ value of [1H]BPA was 52.8 nM, approximately 11% of the binding affinity for the wild-type ERRγ (Table 1). This clearly indicates that the Asn→Gly substitution is greatly disadvantageous for the binding of BPA by ERRγ, probably due to the lack of hydrogen bonding between Asn346 and the phenol hydroxyl group of BPA. The results strongly suggest that it would be difficult to induce BPA to bind to ERRγ.

For the mutant receptors exhibiting a sufficient level of specific binding, the one-site binding hyperbola nonlinear regression analysis of saturation binding curves estimated the receptor density ($B_{max}$, Table 1) for [1H]BPA (Fig. S3), in addition to $K_d$ (Table 1). In the homologous competitive binding assay using tritium-labeled [1H]BPA and non-labeled BPA for the Asn346Gly-ERRγ mutant receptor, almost the same result as described above for [1H]BPA was obtained for non-labeled BPA (Fig. 2C). BPA was clearly weaker in this mutant receptor as compared with the wild-type ERRγ, the $IC_{50}$ value being 42.4 nM (ca. 12% of the binding affinity (4.96 nM) for the wild-type ERRγ) (Table 2).

ERRB has Tyr at position 321 corresponding to Asn346 in ERRγ. When this Tyr was placed at position 346 of ERRγ, the resulting Asn346Tyr-ERRγ exhibited a sufficient specific binding (ca. 60% of the total binding) for [1H]BPA (Fig. S3). The $K_d$ value of [1H]BPA was 31.5 nM, approximately 18% of the binding affinity for the wild-type ERRγ (Table 1). In the competitive binding assay for this ERRγ mutant receptor using [1H]BPA as a tracer, non-labeled BPA exhibited an approximately 9-fold reduction in binding affinity (Table 2). All these results indicate that BPA binds strongly to ERRγ, but not to Asn346Tyr-ERRγ, indicating that the Asn→Tyr substitution at position 346 is disadvantageous for the acceptance of BPA. The structural conversion of the carboxyl amide CONH$_2$ (Asn) into the phenol C$_6$H$_4$OH group (Tyr) is clearly disadvantageous for the binding of BPA.

Construcational importance of Tyr326 as a BPA-binding site

The para-hydroxyl group of Tyr at position 326 is in close proximity to the BPA phenol-B benzene ring. In the BPA/ERRγ-LBD binding complex analysed by X-ray crystallography, the distance between the para-hydroxyl-oxygen atom and benzene-carbon atoms was estimated to be 3.1–4.9 Å in the complex structure [22]. This spacing appeared to allow the intermolecular interaction to a sufficient degree. It should be noted, however, that the Tyr326→Phe mutant receptor lacking the para-hydroxyl group of Tyr-β-phenol was found to be as active as the wild-type [24], indicating that there is no interaction between Tyr326 and the BPA phenol-B benzene ring. This result was somehow surprising, since the two are in extremely close proximity.

The fact that the Phe326 mutant receptor is as active as the wild type with Tyr326 implies definite nonalignment or independence of the BPA benzene B-ring from the Tyr326-para-hydroxyl group. BPA and Tyr326 do not undergo an OH/π interaction, which is one of the strongest molecular interactions. Because the bond angle of C−O−H in Tyr-β-phenol is approximately 105°, the Tyr-β-phenol-para-hydroxyl hydrogen atom must be distant from the BPA benzene B-ring. This causes the Tyr326-β-phenol to act like a wall blocking the pocket from accepting the BPA phenol B-group. This is in stark contrast to the fact that the C2-C3 edge of the Tyr326-β-phenol ring is in close proximity (3.75 Å) to the benzene A-ring of BPA to undergo an edge-to-face π-π interaction, namely, the so-called CH/π interaction [24].

The biphenol A-binding site constructed by the Leu-Asn and Leu-Ile ridges

The binding site of the BPA phenol-B group in ERRγ is made up of Leu342, Leu345, Asn346, and Ile349, being at α-helix H7 of ERRγ-LBD (Fig. 1). Leu342 and Asn346 are on the same (i, i+4) ridge and the other Leu345 and Ile349 are on the adjacent (i+3, i+ 7) ridge in H7. Another important site is Tyr326 on the β-strand 1, and all these five receptor residues construct a binding pocket for the BPA phenol-B group. In order to evaluate the contribution of H7 amino acid residues to the binding of BPA, Leu342, Leu345, Asn346, and Ile349 were mutated to Ala and some other structurally related amino acids.

Leu342. Leu342 was first replaced with Ala. The Leu342Ala-ERRγ mutant receptor exhibited no specific binding for [1H]BPA (Fig. S3), indicating that Leu342 is crucial for the binding of BPA to ERRγ. The Leu342 isobutyl side chain [-CH$_2$CH(CH$_3$)$_2$] was thus clearly essential to hold the BPA’s phenol-B benzene ring. When mutated to Val and Ile, the side chains of which are isopropyl [-CH$_2$CH(CH$_3$)$_2$] and sec-butyl [-CH$_2$CH$_2$CH$_2$CH$_3$], respectively, BPA exhibited a 3–4 fold reduction in the binding affinity for these mutant receptors (Table 1). Similarly, in the homologous competitive binding assay...
of $[^3]$H]BPA/BPA for mutant receptors, BPA showed a 5–7 fold reduction in IC$_{50}$ value (Fig. 2A) (Table 2). These results suggest that the C$_B$-branched side chains are not suitable to accept the BPA molecule at position 342 of ERR$_{\gamma}$.

**Leu345.** In our previous study, we demonstrated that, when BPA binds to ERR$_{\gamma}$ Leu345 makes a back-and-forth rotation of its side chain isopropyl group to adopt a BPA molecule [23]. A strong hydrophobic interaction between the phenol-B benzene ring of BPA and the isopropyl group of Leu345 was eventually demonstrated. The Leu345→Ala substitution, namely, the elimination of the isopropyl group from the Leu side chain $\beta$-methylene, resulted in a drop (approximately 3.5-fold) in the receptor affinity of $[^3]$H]BPA (Table 1). When Leu345 was replaced with Val, the resulting mutant receptor exhibited a sharp drop in its affinity for $[^3]$H]BPA; i.e., an almost 10-fold decrease in the affinity for $[^3]$H]BPA in the saturation binding assay (Table 1) and an approximately 30-fold decrease in the affinity for $[^3]$H]BPA in the $[^3]$H]BPA/BPA homologous competitive receptor binding assay (Fig. 2B) (Table 2).

Although the isobutyl-group of the Leu side chain could potentially make a back-and-forth rotation, the isopropyl group of the Val side chain cannot make such a rotation. Since Val’s isopropyl side chain can rotate freely around the C$_{\alpha}$-C$_B$ axis, it would stay at the most stable location. It is highly likely that this location resulted in a steric hindrance just due to its unfavourable rotatory $\chi$ angle around the C$_{\alpha}$-C$_B$ axis, causing a drastic drop in the receptor affinity of BPA. A similar phenomenon was observed for the Leu345Ile mutant receptor, which showed an approximately 30-fold decrease in the binding of BPA in the homologous competitive binding receptor assay (Fig. 2B) (Table 2). He’s side chain sec-butyl side chain cannot rotate in a back-and-forth manner. All these results indicate that Leu345 is one of the intrinsic determining factors of BPA’s highly specific binding ability for ERR$_{\gamma}$.

**Asn346.** The results of the mutation of Asn346 to Gly and Tyr are described above. In addition, in order to evaluate the structural importance of this Asn346 residue, it was also mutated to Ala. The resulting $[^3]$H]BPA-Ala-ERR$_{\gamma}$ mutant receptor exhibited a fairly high specific binding (ca. 90% of the total binding) for $[^3]$H]BPA (Fig. S3). However, this well-folded Asn346Gln-ERR$_{\gamma}$-LBD protein exhibited a distinctly reduced (2–4 times) binding affinity of BPA (Tables 1 and 2) (Fig. 2C), implying that Asn346’s intrinsic determining factors of BPA’s highly specific binding ability for ERR$_{\gamma}$.

**Ile349.** When Ile349 was replaced with Ala, the resulting Ile349Ala-ERR$_{\gamma}$ exhibited a 3–4 fold reduction in affinity for $[^3]$H]BPA and BPA (Tables 1 and 2). This indicates the importance of the sec-butyl side chain for Ile to bind a BPA molecule. When

### Table 1. Receptor binding characteristics of $[^3]$H]BPA for wild-type ERR$_{\gamma}$ and its mutants in the saturation binding assay.

| Amino acid residues of ERR$_{\gamma}$ receptors$^1$ | Binding characteristics of $[^3]$H]BPA, $K_d$ (nM) | Relative activity (%) | Receptor density for $[^3]$H]BPA, $B_{max}$ (nmol/mg protein) |
|-----------------------------------------------|-----------------------------------------------|---------------------|-----------------------------------------------|
| **Position** | **Mutation** | **Wild-type 5.70±0.88** | **100** | **18.40±0.78** |
| Leu342 | Ala | NSB$^2$ | 0 | NSB |
| | Val | 21.3±2.78 | 27 | 2.87±0.34 |
| | Ile | 14.1±3.31 | 40 | 2.75±0.50 |
| Leu345 | Ala | 19.4±2.04 | 29 | 13.6±0.41 |
| | Ile | 58.1±8.75 | 10 | 4.07±0.21 |
| | Val | 57.3±8.08 | 10 | 7.72±0.55 |
| Asn346 | Ala | 12.3±1.74 | 46 | 18.3±0.69 |
| | Gly | 52.8±6.63 | 11 | 11.8±0.57 |
| | Gln | 19.7±3.20 | 29 | 6.78±0.33 |
| | Tyr | 31.5±6.41 | 18 | 9.37±0.42 |
| Ile349 | Ala | 15.9±0.99 | 36 | 5.92±0.12 |
| | Val | 6.15±1.46 | 93 | 8.84±0.86 |
| | Leu | 9.41±1.12 | 61 | 9.33±1.23 |
| Leu265 | Ala | NSB | 0 | NSB |
| Leu324 | Ala | NSB | 0 | NSB |
| Tyr330 | Ala | 305±20.5 | 2 | 0.91±0.22 |
| Lys430 | Ala | 11.7±1.75 | 49 | 7.37±0.33 |
| His434 | Ala | 8.08±0.11 | 77 | 4.34±0.44 |
| | Phe | 6.63±1.12 | 50 | 6.35±0.57 |

$^1$Specifically mutated residues are designated in italics. All the saturation binding assays to determine the dissociation constant ($K_d$) and the receptor density ($B_{max}$) for $[^3]$H]BPA were carried out at least three times.

$^2$NSB means "no specific binding" in the saturation binding assay.

doi:10.1371/journal.pone.0101252.t001

ERR$_{\gamma}$ Hydrophobic Motif-Binding Pocket for Bisphenol A
Ile349 was replaced with Val and Leu, the activity decrements were rather small: 8-65% for [3H]BPA (Table 1) and 110-135% for BPA (Fig. 2D) (Table 2). However, the importance of Ile349 is beyond doubt, since each of the Ile349Ala-ERRγ, Ile349Val-ERRγ, and Ile349Leu-ERRγ mutant receptors showed clearly lower Bmax values (Table 1).

Collectively, the amino acid residues of Leu342, Leu345, Asn346, and Ile349, which are present at the α-helix H7 of ERRγ, were judged to be authentic binding sites of BPA. Although their interactions with BPA molecules vary considerably, their significance is absolute and fundamental, as evidenced by the site-directed mutagenesis experiments.

Amino acid residues that support the binding sites of BPA’s phenol-B

In our previous study, several amino acid residues were found to assist or structurally support the binding site amino acid residues, the side-chains of which participate in formation of a receptor pocket for the BPA’s phenol-A group [24]. Those included Ile279, Ile310, and Val313. In the present work, we also attempted to explore the surroundings of the amino acid residues of Leu342, Leu345, Asn346, and Ile349, which have been identified as the binding sites of BPA’s phenol-B group. Ala431, which is located within 4.5 Å of the BPA’s sp3 methyl group, was found to be in close proximity to Leu345 (Fig. 3A), and was very likely to provide structural support for the binding site.

In the present study, Val313 was again identified to be important as a structurally supporting residue. When the region surrounding Ile349 was checked carefully, Val313 was found to be an important contribution to the structurally supportive environment (Fig. 3B). In fact, the Ala-substitution of Val313 resulted in a considerable reduction (approximately 5-fold) in the binding affinities of BPA (Table 3). It should be noted that, when the structure of ERRγ-LBD with BPA was compared with the structure of ERRγ-LBD without BPA, the isopropyl-methyl group of Val313 was rotated about 120° to prevent the collision with the BPA’s phenol-A benzene ring [24]. Val313 was thus shown to be one of the key residues for construction of the BPA-binding site in ERRγ-LBD, serving to hold the BPA-binding site amino acid residues in the proper positions. It is of note that there is no direct contact between Val313 and BPA.

Ile310 was found to also be one of the amino acids in the region surrounding Ile349, whereas Leu268 was one of amino acids in the region surrounding Leu342. As reported previously [24], Leu268 is a phenol-B binding site of BPA and is positioned like a clamp or double-hook to tightly connect both of BPA’s phenol-benzene rings. Leu268 was found to be in close proximity to Leu342 (Fig. 3C). In this study, we further found that Leu265 and Tyr330

Figure 2. The homologous competitive binding assays between [3H]bisphenol A and non-labeled bisphenol A for the wild-type ERRγ-LBD and its mutants. The receptors used were the wild-type ERRγ and its mutant receptors. (A) Leu342-substituted ERRγ mutant receptors, (B) Leu345-substituted ERRγ mutant receptors, (C) Asn346-substituted ERRγ mutant receptors, and (D) Ile349-substituted ERRγ mutant receptors. The graphs show representative dose-dependent binding curves, which give the IC50 value closest to the mean IC50 from at least three independent experiments.

doi:10.1371/journal.pone.0101252.g002
were back support residues of Leu268 (Fig. 3D). As a result, Leu268 serves both as a direct BPA-binding site and a back support residue, and Leu265 and Tyr330 serves only as a back support residue. Other newly speculated possible back support residues include Leu324 for Asp346 and Ile349, His434 for Leu342 and Leu345, and Lys430 for Leu345 (Fig. 3). Thus,

Table 2. Receptor binding potency of BPA, 4-α-cumylphenol, and 4-OHT in the competitive binding assay using [3H]BPA for human nuclear receptor ERRγ and its mutants with site-directed mutagenesis in the BPA binding site amino acid residues.

| Amino acid residues of ERRγ receptors | Receptor binding potency IC50 (nM) |
|--------------------------------------|----------------------------------|
|                                       | BPA | 4-α-cumylphenol | 4-OHT |
| Wild-type                            |     |                 |       |
| Leu342                               |     |                 |       |
| Ala                                  | 4.96±0.71 | 5.90±0.30 | 5.87±0.64 |
| Val                                  | 34.7±5.38 | 34.1±0.21 | 25.9±3.50 |
| Ile                                  | 24.5±4.53 | 23.7±0.99 | 16.0±2.87 |
| Leu345                               |     |                 |       |
| Ala                                  | 60.6±11.2 | 87.1±16.6 | 65.3±12.8 |
| Val                                  | 154±17.5 | 122±22.3 | 75.6±15.3 |
| Ile                                  | 158±19.8 | 138±29.8 | 79.0±11.5 |
| Asn346                               |     |                 |       |
| Ala                                  | 20.1±3.41 | 12.9±1.30 | 10.4±0.38 |
| Gly                                  | 42.4±5.44 | 26.2±4.40 | 26.9±2.03 |
| Gln                                  | 56.1±7.42 | 39.9±0.50 | 23.7±1.00 |
| Tyr                                  | 45.2±8.91 | 15.6±1.91 | 22.0±6.65 |
| Ile349                               |     |                 |       |
| Ala                                  | 20.1±3.24 | 19.8±2.20 | 12.7±3.23 |
| Val                                  | 11.7±1.50 | 12.9±0.33 | 12.5±2.29 |
| Leu                                  | 10.4±0.64 | 12.8±1.27 | 13.8±0.79 |

1. Specifically mutated residues are designated in italics.
2. Because there was “no specific binding” in the saturation binding assay, the competitive binding assay could not be carried out.

Table 2. Receptor binding potency of BPA, 4-α-cumylphenol, and 4-OHT in the competitive binding assay using [3H]BPA for human nuclear receptor ERRγ and its mutants with site-directed mutagenesis in the BPA binding site amino acid residues.

| Amino acid residues of ERRγ receptors | Receptor binding potency IC50 (nM) |
|--------------------------------------|----------------------------------|
|                                       | BPA | 4-α-cumylphenol | 4-OHT |
| Wild-type                            |     |                 |       |
| Leu342                               |     |                 |       |
| Ala                                  | 4.96±0.71 | 5.90±0.30 | 5.87±0.64 |
| Val                                  | 34.7±5.38 | 34.1±0.21 | 25.9±3.50 |
| Ile                                  | 24.5±4.53 | 23.7±0.99 | 16.0±2.87 |
| Leu345                               |     |                 |       |
| Ala                                  | 60.6±11.2 | 87.1±16.6 | 65.3±12.8 |
| Val                                  | 154±17.5 | 122±22.3 | 75.6±15.3 |
| Ile                                  | 158±19.8 | 138±29.8 | 79.0±11.5 |
| Asn346                               |     |                 |       |
| Ala                                  | 20.1±3.41 | 12.9±1.30 | 10.4±0.38 |
| Gly                                  | 42.4±5.44 | 26.2±4.40 | 26.9±2.03 |
| Gln                                  | 56.1±7.42 | 39.9±0.50 | 23.7±1.00 |
| Tyr                                  | 45.2±8.91 | 15.6±1.91 | 22.0±6.65 |
| Ile349                               |     |                 |       |
| Ala                                  | 20.1±3.24 | 19.8±2.20 | 12.7±3.23 |
| Val                                  | 11.7±1.50 | 12.9±0.33 | 12.5±2.29 |
| Leu                                  | 10.4±0.64 | 12.8±1.27 | 13.8±0.79 |

Figure 3. Structural interrelationships between the bisphenol A-binding site amino acid residues Leu342-Asn346/Leu345-Ile349 and their back support residues present in the binding pocket of ERRγ-LBD. (A) Leu345 and its back support residue Ala431. There is no direct contact between Ala431 and BPA. (B) Ile349 and its back support residue Val313. There is no direct contact between Val313 and BPA. (C) Leu342 and its back support residue Leu268. (D) Leu268 and its back support residues Leu265 and Tyr330. This Leu268 binds directly to the phenol-A and B rings of BPA as a double-hook to connect both tightly. (E) Ile349/Asn346 and their simultaneous back support residue Leu324. (F) Four amino acids, Leu324, Tyr326, Tyr330, and Met332, stand on the pleated β-sheet, which makes a bottom plate of ERRγ-LBP. (G) Leu345 and its back support residue Lys430. (H) Leu342/Leu345 and their simultaneous back support residue His434.
Leu265, Leu324, Tyr330, Lys430, and His434 were mutated to Ala in order to determine their structural importance in the construction of BPA-binding pockets.

**Leu265.** Leu265 is present in the H3 α-helix, and we speculated that this Leu265 was one of the back support residues of Leu268 (Fig. 3C). As seen for the Leu342Ala-ERRγ mutant receptor, Leu265Ala-ERRγ exhibited no specific binding for $[^3]$H]BPA (Table 1), indicating that Leu265 is critical for the binding to BPA. We could not perform both the saturation and competitive binding assays for $[^3]$H]BPA. Since there is no direct contact between Leu265 and BPA, this Leu265 residue is judged to be a genuine back support residue of Leu268.

**Leu324.** Leu324 is on the S1 β-strand together with Tyr326. The S1 and S2 β-strands form a pleated sheet, which corresponds to a bottom plate of the ERRγ ligand-binding pocket. Leu324 was speculated to be a back support residue of both Asn346 and Ile349 (Fig. 3E), and Leu324Ala-ERRγ exhibited no specific binding for $[^3]$H]BPA (Table 1). These results clearly imply that Leu324 is crucial for ERRγ to bind BPA, particularly as an underlying support of Asn346 and Ile349.

**Tyr330.** Tyr330 is on the S2 β-strand, which forms a pleated sheet together with the S1 β-strand. The resulting pleated β-sheet, bottom plate of the ERRγ-LBP, has four amino acids (Leu324, Tyr326, Tyr330, and Met332) which stand close together inside the pocket (Fig. 3F). In addition to Leu263, we speculated that Tyr330 was a possible back support residue of Leu268 (Fig. 3G). When $[^3]$H]BPA was examined in a saturation binding assay with the Tyr330Ala-ERRγ mutant receptor, $[^3]$H]BPA showed an approximately 5-fold reduction in $K_d$ value (Table 1). In the homologous competitive binding assay of $[^3]$H]BPA/BPA, BPA showed an approximately 3-fold reduction in IC50 value (Table 3). These results imply that the para-hydroxyl group of Tyr330 is an important structural element for the construction of ERRγ-LBP on the bottom pleated β-sheet.

**Lys430.** Lys430 is present in the H11 α-helix, and we speculated that this Lys430 was a back support residue of Lys435 (Fig. 3G). When the Lys430Ala-ERRγ mutant receptor was examined, $[^3]$H]BPA showed only a 40% reduction in $K_d$ value in the saturation binding assay (Table 1), and a 70% reduction in IC50 value in the homologous competitive binding assay of $[^3]$H]BPA/BPA (Table 3). These results suggest that Lys430’s back supporting ability is rather weak as compared to other residues. However, as shown in Table 1, the significance of Lys430 was evidenced by the much lower $B_max$ value of Lys430Ala-ERRγ. These findings suggested that Lys430 is important for the construction of a sound and complete ERRγ-LBP.

When 4-hydroxytamoxifen (4-OHT) was tested in a heterogeneous binding assay using $[^3]$H]BPA for Lys430Ala-ERRγ, it showed a rather stronger receptor binding potency (IC50 = 4.10 nM) as compared with the potency (5.67 nM) for the wild-type ERRγ (Table 3). This reinforced (about 40%) binding potency appears to be due to the structural liberation of Lys435 from Lys430. Ala at position 430 might have been a better support for BPA.

**His434.** His434 is present also in the H11 α-helix, and we speculated that this His434 was a back support residue of Leu342 and Lys435 (Fig. 3H). It was first replaced with Ala, and the resulting His434Ala-ERRγ mutant receptor exhibited no specific binding for $[^3]$H]BPA (Table 1). These results clearly imply that His434 is crucial for the binding of BPA by ERRγ, particularly as a primary support of Leu342 and Lys435. When His was replaced

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**Table 3.** Receptor binding potency of BPA, 4-α-cumylphenol, and 4-OHT in the competitive binding assay using $[^3]$H]BPA for human nuclear receptor ERRγ and its mutants with site-directed mutagenesis in the back support residues of BPA binding sites.

| Amino acid residues of ERRγ receptors | Receptor binding potency IC50 (nM) |
|--------------------------------------|----------------------------------|
| Position                        |       | BPA | 4-α-cumylphenol | 4-OHT   |
| Wild-type                        |       | 5.64±0.17 | 5.72±0.56      | 5.67±0.64 |
| Leu265                           | Ala   | not carried out |
| Leu268                          | Ala   | not carried out |
| Ile310                          | Ala   | 21.9±0.99 | 19.5±2.23      | 20.2±2.33 |
| Val313                          | Ala   | 26.8±2.12 | 21.7±1.88      | 32.5±6.36 |
| Tyr330                          | Ala   | not carried out |
| Lys430                          | Ala   | 16.0±1.41 | 13.5±1.55      | 25.3±1.74 |
| His434                          | Ala   | not carried out |

1)Specifically mutated residues are designated in italics.
2)Because there was “no specific binding” in the saturation binding assay, the competitive binding assay could not be carried out.
3)The results of ERRγ mutant receptors of Leu268, Ile310, and Val313 for BPA, except for those for 4-OHT and 4-α-cumylphenol, were re-collected from the previous report [24] for providing easiness in understanding.

doi:10.1371/journal.pone.0101252.t003
with another aromatic amino acid, Phe, the resulting His434Phe-ERRγ mutant receptor exhibited considerably reduced activities of BPA. When His434Phe-ERRγ was examined, [3H]BPA showed a 100% reduced \( K_d \) value in the saturation binding assay (Table 1), and a 140% reduced IC\(_{50} \) value in the homologous competitive binding assay of [3H]BPA/BPA (Table 3). These results indicate that His434 makes an indispensable contribution to the back support, and thus Phe cannot replace it. The significance of His434 as a primary support of Leu342 and Leu345, and perhaps Phe placed at position 434 provided better back support for these Leu residues, allowing them to accept the phenyl-B benzene ring of 4-\( \alpha \)-cumylphenol. Interestingly, the opposite results were obtained for 4-OHT. 4-OHT exhibited a more than 40-fold reduction in receptor-binding potency (IC\(_{50} = 234 \text{nM} \) for His434Phe-ERRγ as compared with that (5.67 nM) for the wild-type ERRγ (Table 3). It is clear that Phe434 is quite unfavourable for the binding to 4-OHT, but not for the binding to 4-\( \alpha \)-cumylphenol.

**Binding of 4-\( \alpha \)-cumylphenol and 4-hydroxytamoxifen**

In this study we demonstrated that one of the two phenol-hydroxyl groups of BPA makes a hydrogen bond with Asn346. The other phenol-hydroxyl group simultaneously forms hydrogen bonds with Ghu275 and Arg316 [2,23]. 4-\( \alpha \)-Cumylphenol, HO-C\(_6\)H\(_4\)-C(CH\(_3\))-C\(_6\)H\(_5\), lacks one of the two phenol-hydroxyl groups of BPA, and it was found to bind to ERRγ as potently as BPA [2,23]. This immediately raises the question of which hydrogen bond holds 4-\( \alpha \)-cumylphenol in the ligand-binding pocket of ERRγ. The X-ray crystal structure of the complex of 4-\( \alpha \)-cumylphenol/ERRγ-LBD revealed that the phenol-hydroxyl group of 4-\( \alpha \)-cumylphenol makes strong hydrogen bonds with Ghu275/Arg316 of ERRγ, whereas the phenyl group undergoes a hydrophobic interaction with the Leu345-isopropyl group [23]. The fact that 4-\( \alpha \)-cumylphenol is as potent as BPA was thus substantiated by the similarity of their binding modes in the ERRγ-binding pocket. However, one important structural issue arose; that is, 4-\( \alpha \)-cumylphenol did not require the back-and-forth rotation of the Leu345-isobutyl group. In other words, BPA requires the back-and-forth rotation of the Leu345-isobutyl group merely because of the existence of the phenol-B hydroxyl group.

Given these facts, we evaluated the binding activities of 4-\( \alpha \)-cumylphenol for a series of ERRγ mutant receptors. It is clear that all the mutant receptors showed a reduced ability to bind 4-\( \alpha \)-cumylphenol just as observed for BPA (Tables 2 and 3). The Asn346 mutant receptors were the only exception, and their activity decrements observed for 4-\( \alpha \)-cumylphenol were much smaller (1.7–3.4 times) than those for BPA (4.0–11.3 times) (Table 2). Since 4-\( \alpha \)-cumylphenol lacks the BPA’s phenol-B hydroxyl group, which is engaged in a hydrogen bond with Asn346, the smaller affinity drops must have been due to the lack of this hydrogen bond (Fig. 4) (Table 2). It should be noted that Leu345 is the most important among the residues for BPA binding, because the drop in affinity was the largest for the Leu345 mutant receptors (Table 2). This implies that Leu345 is responsible for the recognition followed by the strong affinity binding of BPA’s phenol-B group.

4-Hydroxytamoxifen (4-OHT) works as an inverse agonist, which suppresses the constitutive high activity of ERRγ [1,34,35]. Thus, 4-OHT is a different type of ligand and its binding site appears to be shared only partly with BPA. The molecular volume of 4-OHT is 319.9 \( \text{Å}^3 \), while that of BPA is 187.6 \( \text{Å}^3 \), indicating that the molecular size of 4-OHT is approximately 1.7-fold larger than that of BPA. When 4-OHT was tested in the competitive binding assays for a series of ERRγ mutant receptors with [3H]BPA as a tracer, essentially comparable activity profiles were observed as for BPA (Tables 2 and 3). The results suggested that 4-OHT and BPA share the binding site such as Leu342, Leu345, Asn346, and Ile349 residues on the H7 \( \alpha \)-helix.

**Effects of Ala-substitutions on the constitutive biological activity**

ERRγ is a constitutively active self-activating nuclear receptor, and thus ERRγ exhibits almost the full activity without ligand [1,22,36]. For a series of Ala-substituted ERRγ mutant receptors, the effects of mutations on such constitutive activity were evaluated by means of the luciferase reporter gene assay, and
the results are shown in Fig. 5. When the mutant receptors Leu265Ala-ERRγ, Leu345Ala-ERRγ, Asn346Ala-ERRγ, and Ile349Ala-ERRγ were assayed, Asn346Ala-ERRγ was immediately found to retain approximately 75% of the constitutive activity. In contrast, the other three mutant receptors showed clearly reduced activity: to 30-35% of the activity shown by the wild-type ERRγ (Fig. 5A). These results suggested that a series of amino acids, the pair of Leu265 and Asn346 plus the pair of Leu265 and Ile349, both of which are present in H7, was structurally crucial for the binding of BPA, but the effect of this series on the constitutive activity of ERRγ was rather moderate.

With respect to the constitutive activity, ERRγ must retain its activation conformation, in which α-helix 12 sits on the ligand-binding pocket at an appropriate position. BPA can stay inside the LBP without changing this activation conformation. Most of the amino acid residues inside ERRγ-LBP are simultaneously the binding site of BPA. In addition to Leu265, Leu345, Asn346, and Ile349, the residues Leu268, Leu271, Glu275, Leu309, Arg316, and Tyr326 are also involved. Their Ala-substituted ERRγ mutant receptors were tested previously and the effects of mutations on the constitutive activity were also moderate. Occasionally, however, the Ala-substitutions of back support amino acids were found to cause profound damage to the constitutive activity. For instance, Ile310Ala-ERRγ and Val313Ala-ERRγ were almost completely inactive [24]. This result was also reproduced in the present study as shown in Fig. 5B. Since the conformational damage caused by the Ile310→Ala and Val313→Ala substitutions was found to be irreversible even with a high concentration of BPA, these amino acid residues are crucial for building the activation conformation of ERRγ.

As to other back support amino acids, the Ala-substituted mutant receptors were also tested. The effects of these mutations on the constitutive activity were also moderate, as shown in Fig. 5B. Leu265Ala-ERRγ and Tyr330Ala-ERRγ exhibited approximately 45% and 30% of the constitutive activity of the wild-type ERRγ, respectively, while both Leu324Ala-ERRγ and His343Ala-ERRγ exhibited approximately 70% of the wild-type level of constitutive activity. On the other hand, Lys430Ala-ERRγ showed almost the full constitutive activity of the wild-type ERRγ. All these results clearly indicate that the residual importance of the constitutive activity varies considerably, probably reflecting the difference in structural significance in building of the activation conformation.

**Conclusion**

Based on the results from the X-ray crystal structure analysis of the BPA/ERRγ-LBD complex, we carried out an investigation to identify the binding site of the BPA phenol-B ring, by evaluating the receptor binding and biological activities of Ala-substituted mutant receptors. We confirmed that the amino acid residues Leu268, Leu345, Asn346, and Ile349 form a receptor ligand-binding pocket for the BPA phenol-B ring. We also confirmed that a number of amino acid residues, such as Leu265, Leu268, Ile310, Val313, Leu324, Tyr330, Lys430, Ala431 and His434, functioned as back support residues of these binding sites. The present results reveal that the strong binding of BPA to ERRγ is supported by the double-layer binding sites.

**Supporting Information**

Figure S1 SDS-PAGE elution profiles of the ligand-binding domain (LBD) of wild-type ERRγ and a series of mutants. (A) GST-fused ERRγ-LBD, and (B) GST-free ERRγ-LBD. Three µg of GST-fused ERRγ-LBD and one µg of GST-free ERRγ-LBD expressed proteins were separated on 12.5% SDS-PAGE gel and stained by Coomassie brilliant blue. (TIF)

Figure S2 Temperature-dependent (15–95°C) CD spectra and thermal unfolding curves for representative
GST-free ERRγ-LBD proteins. (A) Wild-type ERRγ, (B) Leu324Ala ERRγ, and (C) Leu342Ala ERRγ. CD spectra in the 200–260 nm UV region are shown by the mean molar ellipticity [θ] in °deg cm²dmol⁻¹. Thermal unfolding curves were depicted, by plotting the mean molar ellipticity [θ] at 222 nm. Ala substitutions of Leu324 (back support residue of Asn346 and Ile349) and Leu342 (direct binding site of BPA’s phenol-B group) resulted in inactivity in binding BPA.

Figure S3 Receptor-binding assays of tritium-labeled bisphenol A (BPA) for the Leu342, Leu345, Asn346, and Ile349 mutant receptors of GST-fused 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 (Kd) and receptor density (Bmax). No Scatchard plot analysis was carried out for the Leu342Ala ERRγ mutant receptor because of its lack of specific binding in the saturation-binding assay. All the saturation binding assays using [3H]BPA were carried out at least three times and a representative result that afforded Kd and Bmax values close to the means is shown for each mutant receptor.

Acknowledgments

We thank Dr. Takeru Nose for comments on early presentations of this work, Dr. Tommaso Costa for comments and discussion on early presentations of this work, and the PLoS ONE reviewers and editorial staff.

Author Contributions

Conceived and designed the experiments: XL AM YS. Performed the experiments: XL. Analyzed the data: XL YS. Contributed reagents/materials/analysis tools: XL AM MS YS. Contributed to the writing of the manuscript: XL YS.

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Acknowledgments

We thank Dr. Takeru Nose for comments on early presentations of this work, Dr. Tommaso Costa for comments and discussion on early presentations of this work, and the PLoS ONE reviewers and editorial staff.

Author Contributions

Conceived and designed the experiments: XL AM YS. Performed the experiments: XL. Analyzed the data: XL YS. Contributed reagents/materials/analysis tools: XL AM MS YS. Contributed to the writing of the manuscript: XL YS.

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