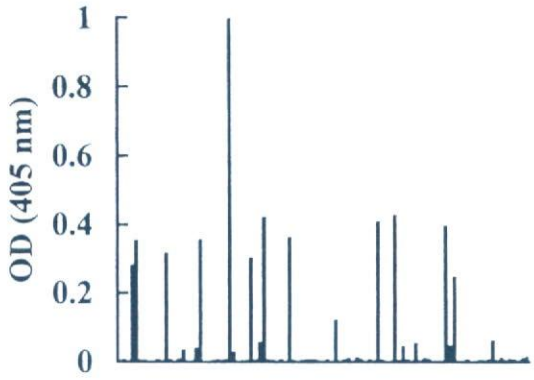

Fig. 2. Screening phage clones in ELISA to evaluate the specific binding to ER-at. The assay was carried out in 10% TFE. A number of clones were found to recognize ER-at, but none of them did bind to ER-LBD.

not contain potential V_HCDR2 and V_HCDR3 regions. The clone 6A was eventually used as a phage-antibody for the conformation-sensing assay.

After titer checking (Fig. 3), competitive ELISA was employed to evaluate the ability of the resulting phage antibody in binding to the ligand-free GR (apo-GR) and ligand-bound GR (holo-GR). Holo-GR was prepared by treatment with 10 μM dexamethasone. It was found that 6A-based phage antibody binds to apo-GR. The capability to differentiate between apo-GR and holo-GR was estimated to be approximately 10%. This result is compatible with our result from the preparation of sensing polyclonal antibody for GR [5].

The present results indicate that the phage display system permit a rapid preparation of monoclonal antibodies (scFvs), which recognize the conformation change of H12. It is essential for bio-pannings to take the conformation of immobilized antigen peptide into consideration.

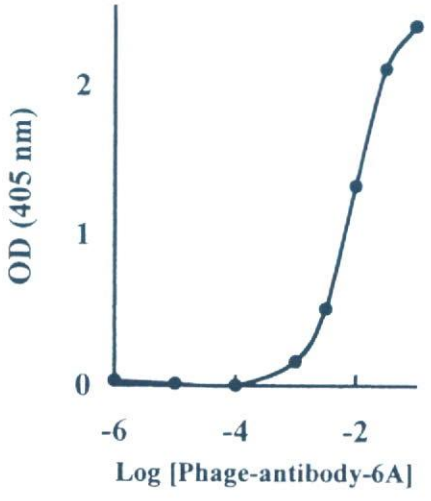

Fig. 3. GR-at1 binding curve for phage-antibody-6A by ELISA. GR-at1 was immobilized at 2.5 μg/ml. GR-at 2 binding curve was depicted almost as above.

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Monoclonal Antibody Sensing Assay for Conformation Change Induced by Metal Binding to Prion Protein N-Terminal Octarepeat Domain

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In the N-terminal region of prion protein, there is a typical repeat structure, in which octapeptide GQPHGGGW is connected in tandem four times. In the present study, we established the method for quantification of the conformation change induced by metal binding by using monoclonal antibody named SAF-32 specific for this octarepeat. SAF-32 exhibited a distinct sensing ability to discriminate the metal ions.

Keywords: conformation change, ELISA, metal binding, monoclonal antibody, prion.

Introduction

Prion protein (PrP^C) is responsible for many transmissible spongiform encephalopathies (TSEs) such as bovine spongiform encephalopathy (BSE) and Creutzfeldt-Jakob disease (CJD). The infectious agent is a misfolded conformational isomer (PrP^{Sc}) of normal cellular prion PrP^C. In the N-terminal region of PrP^C, there is a typical repeat structure in which octarepeat GQPHGGGW is connected in tandem four times. It is well established that this octapeptide repeat (designated as octarepeat hereafter) has an ability to bind to heavy metal ions, especially to copper (II) ion (Cu^{2+}), and this Cu^{2+} binding appears to be one of the most important physiological roles of prion protein. Moreover, it has recently been reported that manganese (II) ion (Mn^{2+}) induces the aggregation of prion protein by binding to prion protein as a substitute for Cu^{2+} [1,2]. Such metal ion coordination should trigger conformation changes of octarepeat region.

In order to quantify the conformation change induced by metal binding to octarepeat, we prepared a monoclonal antibody specific for 24-mer triple-repeated peptide $(GQPHGGGW)_3$ [3]. This antibody was found to bind to the octarepeat, but not to the metal ion-octarepeat complex. The obtained result was found to substantiate the idea that the conformation change occurred can be quantified by estimating the change in immunoresponse of the antibody. Unfortunately, this antibody found not to bind to prion protein *per se*, and thus we intended to utilize a commercially available monoclonal antibody SAF-32 specific for prion protein octarepeat domain [4].

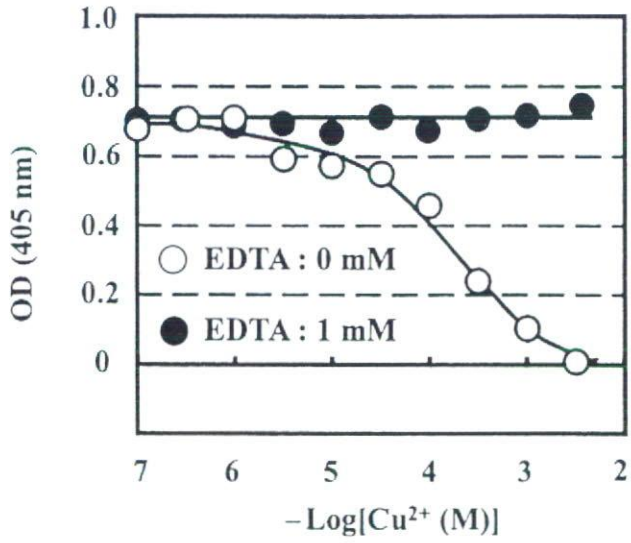

Fig. 1. Binding effect of Cu^{2+} to octarepeat peptide and recovery of the immunoresponse by addition of EDTA.

Results and Discussion

Monoclonal antibody SAF-32 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Binding affinity of this monoclonal antibody to the triple-repeated peptide (OP-3) was confirmed by the ELISA method. The interaction of OP-3 with Cu^{2+} was analyzed in detail by using SAF-32. It was revealed that the immunoresponse of SAF-32 to OP-3 reduces distinctly by the addition of Cu^{2+}, indicating that the conformation of OP-3 changes by binding this divalent ion. The inhibition of immunoresponse took place in a dose-dependent manner of Cu^{2+}, depicting an inhibition curve as shown in Fig. 1. On the other hand, EDTA suppressed this inhibition very sharply and completely (Fig. 1). These results revealed that SAF-32 monoclonal antibody recognizes a certain secondary structure of octarepeat peptide. It is clear that SAF-32 is a useful tool to detect the conformation change of prion protein.

SAF-32 was found to sense the conformation changes induced by other heavy metal ions such as zinc, iron and osmium. The order of binding affinity was $Os^{3+} > Cu^{2+} > Fe^{2+} = Zn^{2+} >> Ni^{2+} = Co^{2+} = Mn^{2+}$. As to Mn^{2+}, the result is contrary to the one reported by others [3,4]. It was found that prion octarepeat does not bind to the Mn^{2+}.

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Conformation Sensing Assay Using Polyclonal Antibody Specific for the C-terminal α-helix of Glucocorticoid Receptor and Progesterone Receptor

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We have prepared polyclonal antibodies that discriminate the ligand-bound and ligand-free conformations of the glucocorticoid receptor (GR) and progesterone receptor (PR). These antibodies were raised against the C-terminal H12 α-helix of GR and PR, which conducts a conformation change along with the ligand binding. Using these antibodies, we established the conformation change-sensing assay that affords the receptor activity parameters corresponding to the receptor binding activity and the hormonal activity.

Keywords: conformation-sensing assay, endocrine disruptors, glucocorticoid receptor (GR), polyclonal antibody, progesterone receptor (PR)

Introduction

Endocrine disruptors are the chemicals that cause the interference or disorder in the endocrine system. Estrogen receptor (ER) is one of the nuclear receptors and a target binding protein of such chemicals. It is known that a number of synthetic chemicals produce undesirable effects on the reproduction system, especially in the process of fetal development in animals and humans. For efficient risk assessment of such undesirable effects, many efforts have been done to develop novel methodolgies. For instance, we did establish a quite novel procedure designated 'conformation-sensing assay.' Preparing a polyclonal antibody that discriminates the conformations of the C-terminal α-helix (designated as H12) of ER, we constructed an assay method to estimate the parameters corresponding to the receptor binding activity and the hormonal activity. H12 is in different positionings, depending upon either the ligand-free or ligand-bound form of ER.

Endocrine disruptors are now aknowledged to have a damaging influence upon not only estrogen receptor, but also all other nuclear receptors including glucocorticoid receptor (GR) and progesterone receptor (PR). Glucocorticoids have a wide range of functions including the regulation of plasma glucose concentration, fat and protein metabolism, and have effects on anti-inflammatory and immunosuppressive actions. On the other hand, progesterone plays an important role in reproductive physiology, as well as bone metabolism and neurotrophic functions. In the present study, we

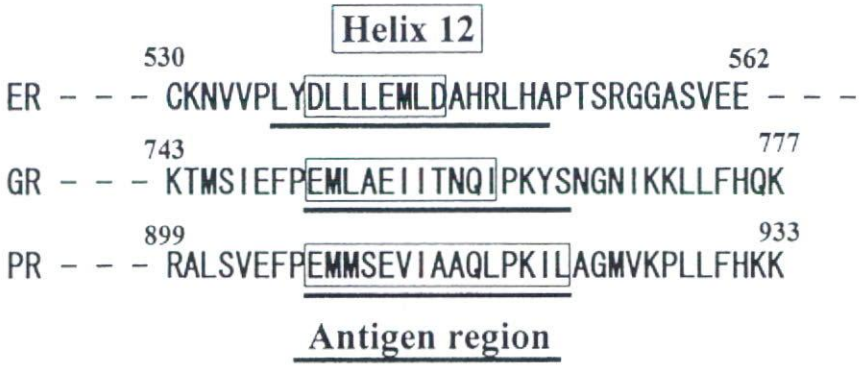

Fig. 1. Peptide fragments selected for preparation of conformation-sensing antibody. Cysteine was attached at the N-terminus of antigen peptides.

attempted to establish the efficient conformation-sensing assay system to endocrine disruptors for GR and PR.

Results and Discussion

To prepare the antibody against H12 in GR and PR, antigen peptides were synthesized by the Fmoc synthetic strategy. To conjugate to career protein KLH, Cys was attached at the N-terminus of each antigen peptide (Fig 1). After immunization into rabbit, the serum collected was first purified by immunoprecipitation with KLH. Further purfication was carried out by affinity chromatograghy using agarose gel conjugating the antigen peptide. Purified antibodies were examined for their ability to bind to the receptor and the target peptide. Both anti-GR and anti-PR antibodies were found to recognize the receptor more strongly than the peptide.

To establish the conformation-sensing assay, bovine thyroglobulin-linked H12-peptide was coated onto the polypropylene 96-well immunoplate as a competiter. Each antibody was examined with or without agonist for the GR and PR, and the agonist used were dexamethasone (Dex) for GR and progesterone for PR. 10 μM of these chemicals were used to construct a ligand-bound holo-form of the receptor. Ligand-free apo-form was examined just without the chemicals. Anti-GR antibody was found to exhibit a sufficient ability to differenciate the apo- and holo-forms of GR (Fig. 2). Also, anti-PR antibody showed such apporopriate immunoresponses under the conditions with or without progesterone (Fig. 2).

At the constant concentration (1 nM for PR, 0.1 nM for GR) of the receptor, the immunoreactivities of each antibody against either the receptor or the peptide fixed on the well was measured under the varying concentrations of chemicals. This was analyzed by plotting the % immunoresponses at each concentration of the chemicals. The maximal immunoresponse R_{max} (%) and the concentration (EC_{50}) to yield a half-maximal immunoresponse $R_{max}/2$ were estimated from this plotting. The R_{max} values were estimated by calculating the immunoresponses relative to the maximal responses of Dex for GR and progesterone for PR. Based on these basal analyses, a series ofchemicals were evaluated their affinity for GR and PR.

As shown in Table 1, cortisol, a natural agonist of GR, exhibited considerably

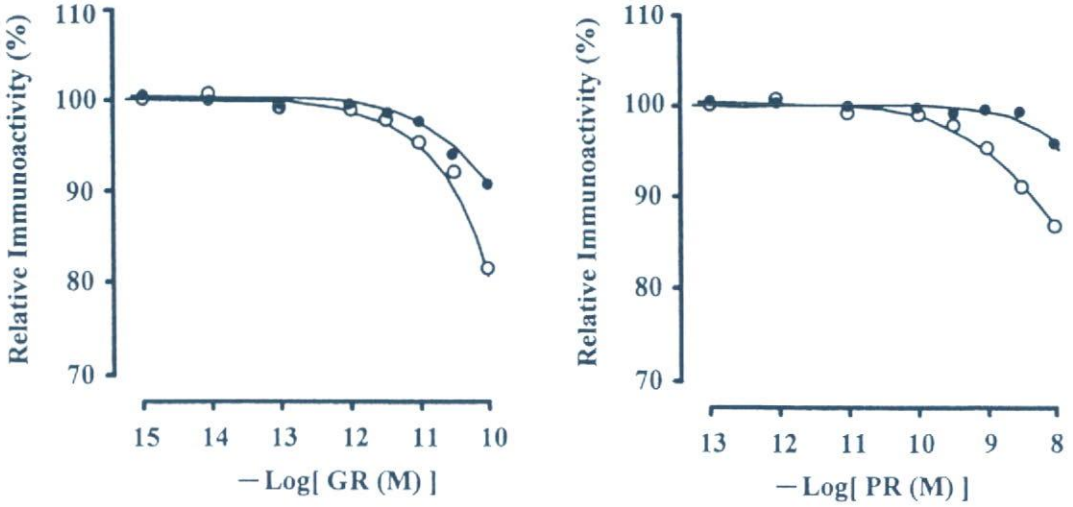

Fig. 2. Immunoresponses of anti-GR (A) and anti-PR (B) antibodies against apo-form (○) and holo-form (λ) of each nuclear receptor. Holo-form was with 10 μM deamethasone (A) and progesterone (B).

Table 1. Immunoresponses of chemicals against glucocorticoid receptor.

Chemicals	EC_{50} (nM)	R_{max} (%)
dexamethasone	32	100
cortisol	860	52
mifepristone	N.B.	N.B.
aldosterone	N.D.	N.D.
cyproterone acetate	N.B.	N.B.

N.B. means that the chemical did not show the immunoresponse. N.D. means that the chemical showed the weak immunoresponse and its EC_{50} value was not able to be determined.

Table 2. Immunoresponses of chemicals against progesterone receptor.

Chemicals	EC_{50} (nM)	R_{max} (%)
norethisterone	33	97
progesterone	87	100
levonorgestrel	220	88
11-ketoprogesterone	N.D.	N.D.
mifepristone	N.B.	N.B.
cyproterone acetate	N.B.	N.B.

N.B. means that the chemical did not show the immunoresponse. N.D. means that the chemical showed the weak immunoresponse and its EC_{50} value was not able to be determined.

weak activity (EC_{50} = 860 nM) as compared to that of Dex (32 nM). Although The result correlated with the data of the receptor binding assay [1], this weak activity may indicate that the antibody obtained is somehow insufficient to discriminate the conformation change induced by cortisol. However, this antibody did not respond to the antagonist mifepristone and cyproterone, indicating that it distinctly discriminates between the agonists and antagonists.

Similar results were also obtained for anti-PR antibody as shown in Table 2. Agonist progesterone was found to be slightly weaker than another agonist norethindrone. Antagonist mifepristone and cyproterone exhibited no responses against this anti-PR antibody. The results clearly show that the antibody discriminates between the agonist- and antagonist-bound conformations of PR.

It is known that PR ligands also bind to androgen receptor (AR) and GR. For example, levonorgestrel was synthesized as a progestin, but it is highly active also to GR and AR [2,3]. Mifepristone is known as antagonist against AR, GR and PR [4,5]. Nonetheless, we could establish the conformation sensing assay for nuclear receptors GR and PR.

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Conformation Change of α-Helix Peptide for Sensing of Deactivation of Nuclear Receptor: Immunoassay Using Polyclonal Antibody Specific for the C-terminal α-Helix 12 of Estrogen-related Receptor γ (ERRγ)

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Introduction

Nuclear receptors (NRs) constitute a large group of transcription factors that are involved in many important biological processes and the regulation of cholesterol metabolism. Forty-eight members of this protein family are known to date in mammals and about thirty possess known ligands or activators [1]. We have recently established the assay system for these NRs to evaluate simultaneously the ligand binding and the hormonal activity of exogenously administered chemicals. This method named as "conformation-sensing assay" requires a specific antibody that discriminates the conformation of ligand-bound holo-NR and ligand-free apo-NR, predominantly at the C-terminal site of α-helix 12 (H12).

Approximately twenty NRs are so-called orphan receptors, the ligands of which are unknown. It is now well acknowledged that some of these NRs retain H12 in an active conformation, resulting in eliciting extremely high basal constitutive activity. Thus, it seemed to be difficult to introduce a sensing assay for such NRs because of their lack of natural ligands. One of such orphan NRs is estrogen-related receptor γ (ERRγ). It was recently found that 4-hydroxytamoxifen (4-OHT) deactivates ERRγ by repositioning its H12 [2], and thus we intended to screen a polyclonal antibody that discriminates this H12's repositioning. In order to evaluate the ability of the chemicals to bind ERRγ, the antibody was utilized to measure their potency in reversing the repositioning of H12 by 4-OHT.

Results and Discussion

To prepare the antibody against H12 in ERRγ, antigen peptide (GKVPMHKLFLEMLEAKV) was synthesized by the Fmoc synthetic strategy. For conjugation to career protein KLH, Cys was attached at the N-terminus of this antigen peptide. After immunization into a rabbit, the serum collected was first purified by immunoprecipitation with KLH. Further purification was carried out by affinity chromatography using agarose gel conjugating the antigen peptide. Purified antibody was found to recognize equally well the receptor and the antigen peptide.

To establish the conformation-change sensing assay, bovine thyroglobulin-linked H12-peptide was coated onto the polypropylene 96-well immunoplate as a competitor. The immunoreactivities of the antibody against the receptor or the immobilized peptide were measured under the varying receptor concentrations. The immunoreactivity of the purified antibody was examined with or without 4-OHT for ERRγ. 10 μM 4-OHT was applied to construct a holo-form of the receptor, and the reactivity of the apo-fprm was tested just without the chemical. Anti-ERRγ-H12 antibody was found to exhibit a sufficient ability to differentiate the apo- and holo-forms of ERRγ (Fig. 1).

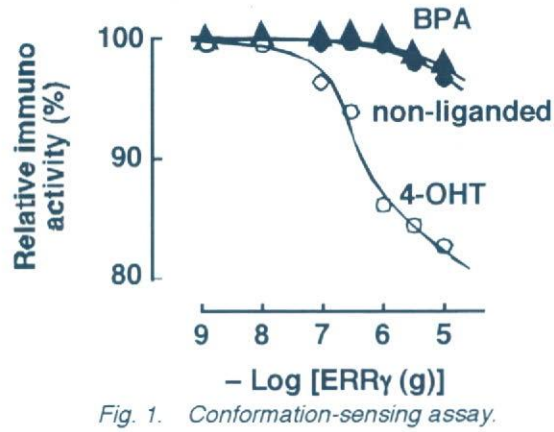

Fig. 1. Conformation-sensing assay.

H12 in 4-OHT-bound ERRγ does not occupy the coactivator groove and shifts from the constitutive-active position [3]. It was thought that prepared anti-H12 polyclonal antibody can distinguish this position change, recognizing 4-OHT-bound ERRγ better than ligand-free ERRγ.

Bisphenol A (BPA) was found to bind to ERRγ very strongly in the receptor binding assay. In the reporter gene assay, BPA retained a high constitutive activity of ERRγ. 4-OHT deactivates ERRγ in a dose-dependent manner, but BPA was found to reverse this 4-OHT's inverse agonist activity. This reversal effect of BPA on ERRγ was also demonstrated in the conformation change sensing assay.

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Functional Analysis of F-domain Peptides Important for the Basal Constitutive Activity of Human Nuclear Receptor

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Introduction

Nuclear receptors (NRs) are essential transcriptional regulators involved in widely diverse physiological functions such as control of embryonic development, cell differentiation, and homeostasis. 48 human NRs are classified into seven groups (NR1-NR6 and NR0) along with their homology of amino acid sequences. These receptor proteins possess structurally highly conserved domains; a DNA binding domain in the zinc finger motif structure (DBD), and a ligand binding domain (LBD) (Fig. 1). LBD is in the α domain structure, which is also highly conserved to retain NRs in a common molecular mechanism as a transcription factor. Interestingly, we found that there are several NRs lacking the F-domain completely or partly, in spite of the presence of NRs having very long F-domain peptides. Apparently, this appeared to relate with their basal levels of constitutive activity.

Approximately 30 NRs possess distinct ligands or activators, while others are orphan receptors, the ligand of which is unknown. Some of orphan receptors are believed not to have an endogenous ligand. To convey different transcription activities, these NRs might evolve especially for ligand recognition, while the structure essential for transcription mechanism might be maintained. In this study, to demonstrate the structural requirement for all human NRs or specific for each NR group, we analyzed structural essentials by means of the evolutionary trace (ET) method. ET is an algorithm that proves the evolutionary record that resides in the divergent amino acid sequences of a large gene family and determines the importance of each amino acid position by correlating their variations with evolutionary divergences [1,2].

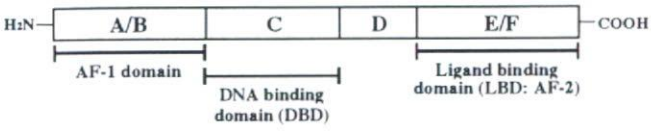

Fig. 1. The basic structure of nuclear receptors

Results and Discussion

When we applied the ET method for all of 48 NR-LBD, thirty amino acid residues were found to be preserved. We then mapped and highlighted them onto the 3D-structures of four different NRs, namely the representatives of NR1-NR4 groups. Those include TRβ (PDB-code = 1XZX), RXRα (1FBY), ERα (1GWR), and Nurr1 (1OVL). Two distinct clusters were clearly evidenced for all four NRs. One is a cluster in the interior of coactivator interaction site, and the other is a cluster in the interior of dimer interface. Both sites are highly conserved among almost all NRs, suggesting that the coactivator interaction and the dimer formation are crucially important for their basal functions.

In interior of dimmer interface, TRβ has the Arg residue at position 429, where its guanidino group makes a hydrogen bond with Ser380. This interaction would stabilize strongly the dimer interface, but apparently only in the NR1 group. In other NR groups, the corresponding Arg side chain orientates to an opposite direction. In coactivator binding site, Glu422 in Nurr1 is specific for NR4 group. Glu422 makes a hydrogen bond with Arg418 backbone, and this interaction would stabilize the coactivator binding site to retain a high constitutive activity. Other NR groups possess Lys instead of Glu at the position.

The F-domain is a C-terminal tail moiety tethered to helix-12, and constitutes a part of coactivator interaction site. It cannot be analyzed by the ET method, because the amino acid sequences of F-domain are completely different from each other. To obtain the information about structure-activity relationship (SAR), we attempted to catalog by aligning all NRs by their lengths. A good linear correlation was found between the size of F-domain peptide fragment and the level of basal constitutive activity. All these results strongly suggested that the molecular function of NRs is largely dependent upon their ordered structures in the C-terminal moiety.

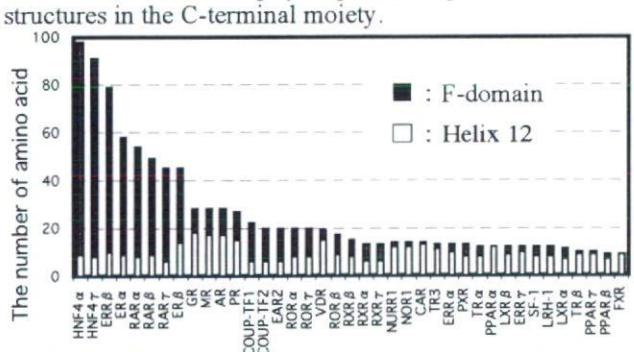

Fig. 2. The number of amino acid in H12 and F-domain

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Receptor binding characteristics of the endocrine disruptor bisphenol A for the human nuclear estrogen-related receptor γ

Chief and corroborative hydrogen bonds of the bisphenol A phenol-hydroxyl group with Arg316 and Glu275 residues

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Keywords
bisphenol A; estrogen-related receptor γ; nuclear receptor; receptor binding site; receptor binding assay

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Bisphenol A, 2,2-bis(4-hydroxyphenyl)propane, is an estrogenic endocrine disruptor that influences various physiological functions at very low doses, even though bisphenol A itself is ineffectual as a ligand for the estrogen receptor. We recently demonstrated that bisphenol A binds strongly to human estrogen-related receptor γ, one of 48 human nuclear receptors. Bisphenol A functions as an inverse antagonist of estrogen-related receptor γ to sustain the high basal constitutive activity of the latter and to reverse the deactivating inverse agonist activity of 4-hydroxytamoxifen. However, the intrinsic binding mode of bisphenol A remains to be clarified. In the present study, we report the binding potentials between the phenol-hydroxyl group of bisphenol A and estrogen-related receptor γ residues Glu275 and Arg316 in the ligand-binding domain. By inducing mutations in other amino acids, we evaluated the change in receptor binding capability of bisphenol A. Wild-type estrogen-related receptor γ-ligand-binding domain showed a strong binding ability (K_D = 5.70 nM) for tritium-labeled [^{3}H]bisphenol A. Simultaneous mutation to Ala at positions 275 and 316 resulted in an absolute inability to capture bisphenol A. However, individual substitutions revealed different degrees in activity reduction, indicating the chief importance of phenol-hydroxyl↔Arg316 hydrogen bonding and the corroborative role of phenol-hydroxyl↔Glu275 hydrogen bonding. The data obtained with other characteristic mutations suggested that these hydrogen bonds are conducive to the recruitment of phenol compounds by estrogen-related receptor γ. These results clearly indicate that estrogen-related receptor γ forms an appropriate structure presumably to adopt an unidentified endogenous ligand.

Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane, has long been recognized as an estrogenic chemical able to interact with human estrogen receptor (ER) [1–3], and recently was reported also to act as an antagonist for a human androgen receptor (AR) [4,5]. In addition, various so-called 'low-dose effects' of BPA have been reported *in vivo* for many organ tissues and systems in mice and rats [6,7]. Because the binding of

Abbreviations
BPA, bisphenol A; ER, estrogen receptor; ERR, estrogen-related receptor; ERRE, ERR-response element; ERRγ, estrogen-related receptor γ; GST, glutathione *S*-transferase; LBD, ligand-binding domain; NR, nuclear receptor; 4-OHT, 4-hydroxytamoxifen.

BPA to ER and AR and its hormonal activity is extremely weak (1000–10 000-fold weaker than for natural hormones), it is unlikely that BPA interacts directly with ER and AR to achieve these effects at low doses [8–11].

Based on the idea that BPA may interact with nuclear receptors (NRs) other than ER and AR, we searched a series of NRs and eventually succeeded in exploring a target NR of BPA [12]. BPA was found to bind strongly to estrogen-related receptor γ (ERRγ), one of 48 human NRs [13], with high constitutive basal activity. We found that BPA inhibits the inverse agonist activity of 4-hydroxytamoxifen (4-OHT), which deactivates ERRγ in, for example, the luciferase reporter gene assay. BPA reverses such deactivation to the originally high basal activation state in a dose-dependent manner, and thus acts as an inverse antagonist of ERRγ.

ERRs are a subfamily of orphan NRs and are closely related to two ERs: ERα and ERβ [14,15]. The ERR family includes three members (ERRα, ERRβ, and ERRγ) with ERRγ being the most recently identified member [16–18]. Amino acid sequences are considerably conserved among ERRs and ERs, especially in their DNA-binding domain and ligand-binding domain (LBD). However, 17β-estradiol, a natural ligand of ERs, does not bind to any members of the ERR family [14,19]. Likewise, BPA binds only weakly to ERs and does not bind at all to any other receptors of the ERR family.

BPA has the chemical structure HO-C_6H_4-$C(CH_3)_2$-C_6H_4-OH, with two phenol groups and two methyl groups on the sp^3 tetrahedral carbon atom (Fig. 1). We recently carried out crystallization and X-ray structural analysis of the BPA/ERRγ-LBD complex [20]. In the complex, a single molecule of BPA stays at the ligand-binding pocket of each ERRγ-LBD protein molecule, the α-helix 12 (H12) of which is stabilized in an activation conformation. The crystal structure of the complex suggests that several essential interactions occur between the BPA and ERRγ-LBD molecules. For example, the phenol-hydroxyl group of BPA is tethered by hydrogen bonds to the Glu275 and Arg316 residues in the ERRγ-LBD (Fig. 2).

For a better understanding of the basal binding potentials to capture a putative endogenous ligand in a ligand-receptor binding pocket, it is crucial to clarify the structural requirements for ligand(s), if any. In the present study, to shed light on the structural elements of ERRγ, we carried out a site-directed point mutagenesis series for the candidate amino acid residues in ERRγ-LBD. We report that the Glu275 and Arg316 residues of ERRγ-LBD are structurally essential for capturing conjunctively the phenol-hydroxyl group of BPA.

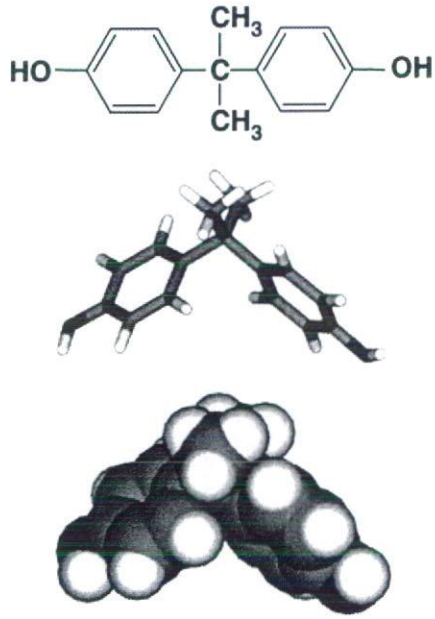

Fig. 1. Chemical structure BPA and its ball-and-stick structure, together with a space-filling structure in the ligand-binding pocket of the ERRγ. The space-filling structure of BPA originated from the X-ray crystal structure (Protein Data Bank with accession code 2E2R) [20].

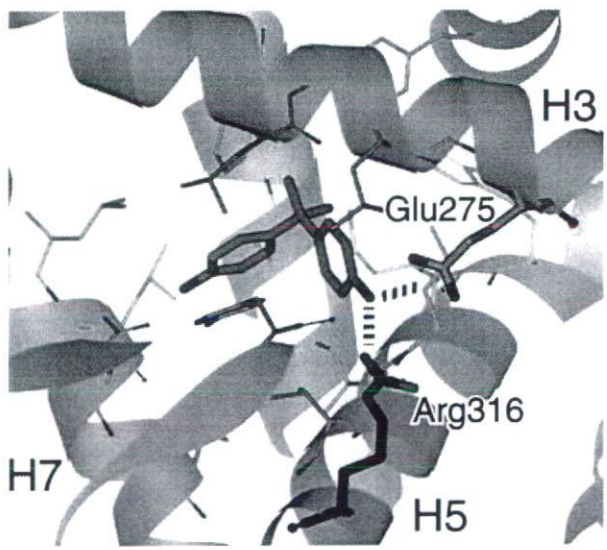

Fig. 2. Structural environments of BPA in the ligand-binding pocket of the ERRγ. The proximity of each amino acid residue (within a distance of 5 Å) to BPA is shown in the boxes depicting the α-helices. The portrait was originated from the X-ray crystal structure (Protein Data Bank with accession code 2E2R) [20].

Results

Deactivation by simultaneous Ala substitution of Glu275 and Arg316

For the receptor binding assays, the LBD of ERRγ was expressed in *Escherichia coli* as a protein fused with glutathione *S*-transferase (GST). A cDNA fragment encoding wild-type ERRγ-LBD (residues 222–458) was generated by PCR from the human kidney cDNA library and cloned into the vector for GST fusion. Mutations were introduced by the PCR mutagenesis method [21], and sequence accuracy was confirmed for each mutant. Site-directed mutations were carried out for positions 275 and 316, the original amino acids for which are Glu (= GAG) and Arg (= CGG), respectively.

Saturation binding assay was performed by using GST-ERRγ-LBD and tritium-labeled [^{3}H]BPA. Specific binding of this [^{3}H]BPA was calculated by subtracting the nonspecific binding (with 10 μM BPA) from the total binding. Figure 3A shows the results of saturation binding assays using [^{3}H]BPA and the wild-type ERRγ receptor, depicting a sufficient specific binding activity (77%).

To demonstrate the suggestion that the phenol-hydroxyl group of BPA is engaged in hydrogen bonds with the Glu275 and Arg316 residues in the ERRγ-LBD [20], these residues were simultaneously mutated to Ala. As shown in Fig. 3D, the resulting (*Ala*, *Ala*)-ERRγ mutant receptor did not exhibit a specific binding sufficient for further analysis. In case no specific binding was measurable under the same experimental conditions for the wild-type ERRγ receptor, the assay was repeated a certain number of times using various concentrations of the receptor or radio ligand. Eventually, we found only nonspecific binding for (*Ala*, *Ala*)-ERRγ without any specific binding, as preliminarily reported [20] (Fig. 3D).

The results clearly indicate that Glu275 and Arg316 are crucial for the binding of BPA, and thus their side chain carboxyl and guanidino groups are indeed engaged in hydrogen bonding with the phenol-hydroxyl group of BPA (Fig. 2). The phenol-hydroxyl group (-OH) has a proton-donating character as well as a proton-accepting character. Thus, it is easy to bridge by hydrogen bonding between the phenol-hydroxyl group of BPA and both the Glu275 and Arg316 residues.

Differential ability of Glu275 and Arg316 in making hydrogen bonds to hold BPA in the binding pocket

Dissociation constants of [^{3}H]BPA from the saturation binding assays

Because both Glu275 and Arg316 were involved in the hydrogen bonding with BPA, we attempted to examine which hydrogen bond most strongly holds BPA in the ligand-binding pocket of ERRγ. Thus, these amino acid residues were mutated independently to Ala. When the Glu275 → Ala substitution was

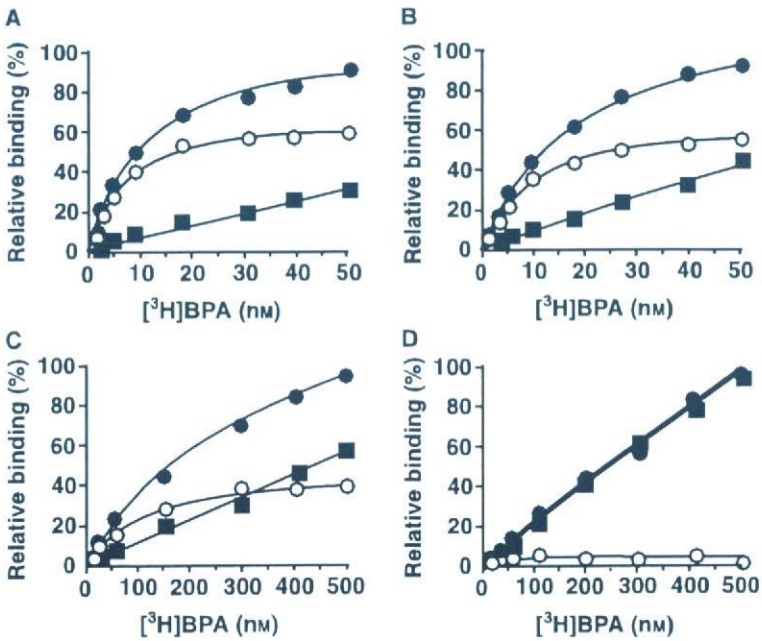

Fig. 3. Saturation binding curves from the radioligand receptor binding assay for the ERRγ by BPA. Saturation binding curves were attained for [^{3}H]BPA for the recombinant human ERRγ LBD and its site-directed mutant derivatives. The graphs show total (●), specific (○), and nonspecific (■) bindings. Determination of nonspecific binding was carried out by an excess of unlabeled chemical (10 μM). (A) Wild-type ERRγ, (B) (275*Ala*)-ERRγ with the Glu275 → Ala substitution, (C) (316*Ala*)-ERRγ with the Arg316 → Ala substitution, and (D) (*Ala*, *Ala*)-ERRγ with simultaneous Glu275 → Ala and Arg316 → Ala substitutions.

accomplished, the resulting mutant receptor (275*Ala*)-ERRγ was found to exhibit sufficient specific binding (approximately 55% of the total binding) for [^{3}H]BPA (Fig. 3B). In addition, (316*Ala*)-ERRγ with the Arg316 → Ala substitution exhibited barely sufficient specific binding (approimately 40% of the total binding) for [^{3}H]BPA (Fig. 3C), although much higher concentrations of [^{3}H]BPA were required.

When the Glu275 → Ala substitution was accomplished, the resulting mutant receptor (275*Ala*)-ERRγ was found to exhibit considerably decreased binding potency for BPA. Given the absence of a carboxymethyl group of Glu275, the binding energy of [^{3}H]BPA to (275*Ala*)-ERRγ was estimated to be considerably weaker than that to wild-type ERRγ. Indeed, it showed significantly diminished binding ability with a dissociation constant of 17.8 nM (32% of the binding affinity for the wild-type ERRγ) (Fig. 4, Table 1).

The Arg316 → Ala substitution resulted in a further diminution of activity (Fig. 4). The dissociation constants were 171 nM (only 3.3% of the binding affinity for the wild-type ERRγ) for [^{3}H]BPA (Fig. 4, Table 1). These results clearly indicate that the hydrogen bonds between the phenol-hydroxyl group of BPA and the Glu275 and Arg316 residues are crucial for capturing BPA in the binding pocket of the ERRγ-LBD. Moreover, it is clear that the hydrogen bond between the BPA and Arg316 is much more important than that between BPA and the Glu275.

Table 1. Receptor binding characteristics of ERRγ and its mutants by [^{3}H]BPA. Specifically mutated residues are shown in italics. NSB, no specific binding in the saturation binding assay.

Amino acid residues of ERRγ receptors		Binding characteristics of [^{3}H]BPA	
Position 275	Position 316	Dissociation constant (K_D, nM)	Receptor density (B_{max}, nmol/mg)
Glu	Arg[a]	5.70 ± 0.88	18.4 ± 0.78
Ala	Arg	17.8 ± 2.74	6.72 ± 0.62
Asp	Arg	22.0 ± 2.86	12.4 ± 0.46
Gln	Arg	23.4 ± 3.34	7.81 ± 0.47
Leu	Arg	NSB	NSB
Glu	*Ala*	171 ± 39.5	0.56 ± 0.09
Glu	*Lys*	22.5 ± 4.26	9.98 ± 0.76
Glu	*Leu*	NSB	NSB
Ala	*Ala*	NSB	NSB
Arg	*Glu*	59.7 ± 6.79	3.66 ± 0.29
Ala	*Glu*	NSB	NSB
Arg	*Ala*	54.3 ± 6.82	3.56 ± 0.38

[a]Wild-type.

Binding affinity of BPA and 4-OHT in competitive receptor binding assays

The receptor binding results obtained here were also revealed by a competitive binding assay, using [^{3}H]BPA as a tracer. We tested the nonradio-labeled BPA and 4-OHT to evaluate their ability to displace [^{3}H]BPA in the ERRγ ligand-binding pocket. The phenol-hydroxyl group of 4-OHT, an estrogen receptor modulator, shares the same site for its binding to ERRγ [20,22]. BPA and 4-OHT elicited almost the same strong binding activity for the wild-type ERRγ (Table 2, Fig. 5). On the other hand, the concentrations for half-maximal inhibition (IC_{50}) of BPA were 35.7 nM for (275*Ala*)-ERRγ, 27% of the binding affinity for the wild-type ERRγ, and 990 nM for (316*Ala*)-ERRγ, only approximately 1% of that for the wild-type (Fig. 5A, Table 2). The values of IC_{50} and K_D essentially reveal their inter-relationship.

The IC_{50} values of 4-OHT were 53.2 nM for (275*Ala*)-ERRγ (25% of that for the wild-type) and 818 nM for (316*Ala*)-ERRγ (1.6%) (Fig. 5B, Table 2). These results indicate clearly that the hydrogen bonding to the Arg316 residue is more important for capturing BPA and 4-OHT than is the bonding to the Glu275 residue in the binding pocket of ERRγ-LBD.

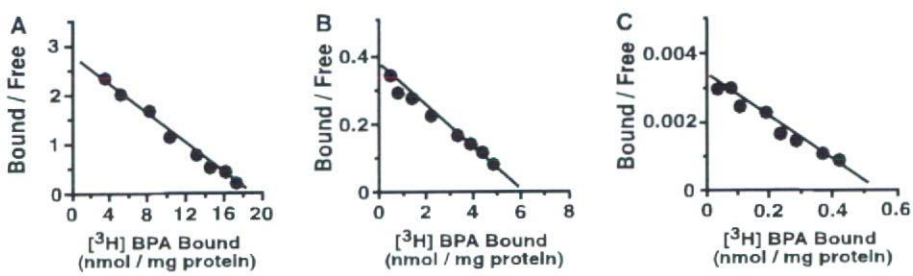

Fig. 4. Scatchard plot analyses showing a single binding mode with a binding affinity constant (K_D) and receptor density (B_{max}). Analyses were carried out from the radioligand receptor saturation binding curves of [^{3}H]BPA for the human ERRγ LBD and its site-directed mutant derivatives. Those include the wild-type ERRγ (A), (275*Ala*)-ERRγ with the Glu275 → Ala substitution (B), and (316*Ala*)-ERRγ with the Arg316 → Ala substitution (C).

Table 2. Receptor binding potency of BPA and 4-OHT in the competitive binding assay for ERRγ and its mutants by [^{3}H]BPA. Specifically mutated residues are shown in italics. Because there was no specific binding in the saturation binding assay, the competitive binding assay could not be carried out. ND, Not determined.

Amino acid residues of ERRγ receptors		Receptor binding potency IC_{50} (nM)	
Position 275	Position 316	BPA	4-OHT
Glu	Arg[a]	9.70 ± 0.59	13.3 ± 3.02
Ala	Arg	35.7 ± 5.48	53.2 ± 10.8
Asp	Arg	36.7 ± 7.18	49.3 ± 8.65
Gln	Arg	52.1 ± 8.99	37.1 ± 5.74
Leu	Arg	ND	ND
Glu	*Ala*	990 ± 184	818 ± 105
Glu	*Lys*	37.1 ± 4.73	54.9 ± 11.3
Glu	*Leu*	ND	ND
Ala	*Ala*	ND	ND
Arg	*Glu*	195 ± 24.5	200 ± 28.8
Ala	*Glu*	ND	ND
Arg	*Ala*	154 ± 32.5	243 ± 17.7

[a]Wild-type.

When Glu275 and Arg316 were each replaced by Leu instead of Ala, the resulting (275*Leu*)-ERRγ and (316*Leu*)-ERRγ mutant receptors were completely inactive, with no specific binding (Table 1). Thus, it was impossible to carry out competitive binding assays for them (Table 2). Because Leu has an additional $-CH(CH_3)_2$ (= isopropyl) group on the β-carbon of the Ala side chain, this hydrophobic bulky group is apparently disadvantageous electrochemically and/or spatially for the interaction with BPA or 4-OHT. Glu has the $-CH_2COOH$ (carboxymethyl) group on the β-carbon of the Ala side chain, whereas Arg has $-CH_2CH_2NHCH(=NH)NH_2$. These groups are capable of making hydrogen bonds with the phenol-hydroxyl group of BPA and also with that of 4-OHT, providing the space that fits the phenol group perfectly.

Replacement of Glu275 and Arg316 with structurally similar amino acids

When Glu275 was replaced solely by glutamine (Gln), with the substitution of the γ-carboxyl (COOH) of Glu to carboxyl amide ($CONH_2$), the resulting (275*Gln*)-ERRγ mutant receptor exhibited a sufficient level of specific binding (approximately 70% of the total binding) for [^{3}H]BPA (data not shown). The K_D values were 23.4 nM (approximately 25% of the binding affinity for the wild-type ERRγ) (Table 1). The IC_{50} values of BPA and 4-OHT were 52.1 nM (19% of the binding affinity for the wild-type) and 37.1 nM (36%), respectively (Table 2). These results are almost equal to those obtained for (275*Ala*)-ERRγ. Thus, the Gln-carboxyl amide ($CONH_2$) group cannot replace the Glu-carboxyl (COOH) group.

In addition to the previous finding, (275*Asp*)-ERRγ with the Glu275 → Asp substitution exhibited a sufficient level of specific binding (approximately 70% of the total binding) for [^{3}H]BPA (data not shown). This mutant receptor (275*Asp*)-ERRγ exhibited only moderate activity levels (30–50%) for BPA and 4-OHT, however, which were similar to those obtained for (275*Ala*)-ERRγ (Tables 1 and 2). Asp with the β-carboxyl group is an acidic amino acid, like Glu, but it lacks the methylene group (CH_2) of Glu at the γ position. All these results indicate that the substitutions of Glu275 with Gln and Asp, and even with Ala, decrease considerably the binding ability of BPA and 4-OHT, but do not cause inactivity. It is evident that only Glu275 can elicit full activity, as long as the Arg316 residue is retained.

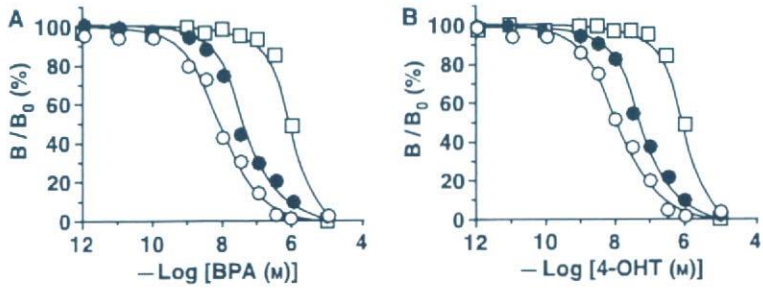

Fig. 5. Receptor competitive binding assays for the ERRγ and its mutants using [^{3}H]BPA. The assays were carried out to measure the ability to displace [^{3}H]BPA for wild-type ERRγ (○), (275*Ala*)-ERRγ with the Glu275 → Ala substitution (●), and (316*Ala*)-ERRγ with the Arg316 → Ala substitution (□). Chemicals used are BPA (A) and 4-OHT (B). The graphs show representative dose-dependent binding curves, which give the IC_{50} value closest to the mean IC_{50} from at least five independent assays. The IC_{50} values showed a between-experiment coefficient of variation of 4–9%. All the receptors used are the LBD of the human ERRγ and its mutant receptors.

The inactivity of (316*Leu*)-ERRγ and the extremely weak activity of (316*Ala*)-ERRγ (Tables 1 and 2) definitely reveal the importance of the basic Arg residue for receptor activation. Instead of Arg with the guanidino -NH-CH(=NH)NH_2 group, there is Lys with the amino group. Prepared (316*Lys*)-ERRγ was found to be considerably potent for binding [^{3}H]BPA (K_D = 22.5 nM) (Table 1). In the competitive binding assay using (316*Lys*)-ERRγ and [^{3}H]BPA, BPA was significantly active (IC_{50} = 37.1 nM) (Table 2). However, these activities are only approximately 25% that of the parent wild-type receptor ERRγ. Collectively, these results indicated that Arg316 is the most important structural element for the binding of BPA and 4-OHT to the binding pocket of ERRγ-LBD by hydrogen bonding.

Residual exchange between Glu275 and Arg316 keeps BPA in a binding pocket

It is now clear that Glu275 and Arg316 are necessary to hold BPA and 4-OHT in ERRγ, but with different degrees of involvement in the hydrogen bonding. The results clearly indicated the chief importance of phenol-hydroxyl↔Arg316 hydrogen bonding, whereas a corroborative role was indicated for the phenol-hydroxyl↔Glu275 hydrogen bonding. Given that the roles of these residues definitely confirm each other, the difference in their significance might be attributable to the importance and/or necessity of the receipt of the phenol-hydroxyl group, even by using an assisting group to facilitate the receptor function. No other amino acids would reward such an intrinsic role of a combination of 316Arg and 275Glu.

Thus, if we simply put these residues in opposite order, the resulting (*Arg*, *Glu*)-ERRγ double-mutant receptor would be exchangeable, but would have considerably lower affinity to BPA and 4-OHT. The mismatched proximity of Arg275 and Glu316 to the phenol-hydroxyl group of BPA and of 4-OHT would take place because an unchanged backbone structure is strongly suspected for α-helix-rich ERRγ-LBD. Indeed, these chemicals were found to bind to the (*Arg*, *Glu*)-ERRγ double-mutant receptor. However, as expected, they bound to the receptor approximately ten-fold more weakly than to the wild-type receptor (Tables 1 and 2).

Although Glu275 and Arg316 in ERRγ were found to be exchangeable for maintaining the interaction with BPA and 4-OHT (Table 2), their ability either to hold or have a role in retaining the phenol compounds in the resulting (*Arg*, *Glu*)-ERRγ receptor might be the same as that for the wild-type ERRγ. Further substitution of 275*Arg* and 316*Glu* with Ala resulted in a similar outcome: the chief role of phenol-hydroxyl↔275*Arg* hydrogen bonding and a corroborative role of the phenol-hydroxyl↔316*Glu* hydrogen bond. (*Ala*, *Glu*)-ERRγ mutant receptor with the 275*Arg* → Ala substitution was found to completely lack the binding capability for [^{3}H]BPA, whereas the Arg-containing (*Arg*, *Ala*)-ERRγ mutant receptor was still active (Table 1). It should be noted that (*Arg*, *Glu*)-ERRγ is almost equipotent with (*Arg*, *Ala*)-ERRγ (Table 1). This indicates that the corroborative role of the phenol-hydroxyl↔316*Glu* hydrogen bond is almost negligible. As a result, the wild-type ERRγ receptor appears to afford simultaneously an ideal space and the capability of arresting the phenol-hydroxyl groups by arranging the Glu and Arg residues at positions 275 and 316, respectively.

Evaluation of the basal constitutive activity of ERRγ mutant receptors

We examined the biological activity of BPA in the reporter gene assay in HeLa cells transiently cotransfected with an ERRγ receptor expression plasmid and an ERR response element (ERRE)-luciferase reporter plasmid. For reference estimations, the cells were treated with a vehicle solution to measure the basal constitutive activity of each receptor, by using exactly the same of amount of expression plasmid of the receptor. Furthermore, to normalize for transfection efficiency, we carried out simultaneously a SEAP assay [23], in which we cotransfected a second plasmid that constitutively expresses an activity that can be clearly differentiated from SEAP.

When we compared ERRγ mutant receptors with wild-type ERRγ, we found the constitutive activity levels differed considerably. As shown in Fig. 6A, the (275*Ala*)-ERRγ mutant receptor exhibited moderately elevated constitutive activity (42% of the basal activity of wild-type ERRγ). However, the (316*Ala*)-ERRγ mutant receptor with the Arg → Ala substitution exhibited considerably diminished constitutive activity (25%), and (*Ala*, *Ala*)-ERRγ became very weak (9%). These results clearly show that both Glu275 and Arg316, especially the latter residue, are important for constructing a high level of basal activity.

The wild-type ERRγ is fully activated spontaneously with no ligand. BPA (10^{-10} to 10^{-5} M) sustains this high level of ERRγ basal constitutive activity (Fig. 6B), as reported previously [12]. By contrast, BPA exhibited an extremely weak tendency to activate the mutant receptors of (275*Ala*)-ERRγ and (316*Ala*)-ERRγ in a

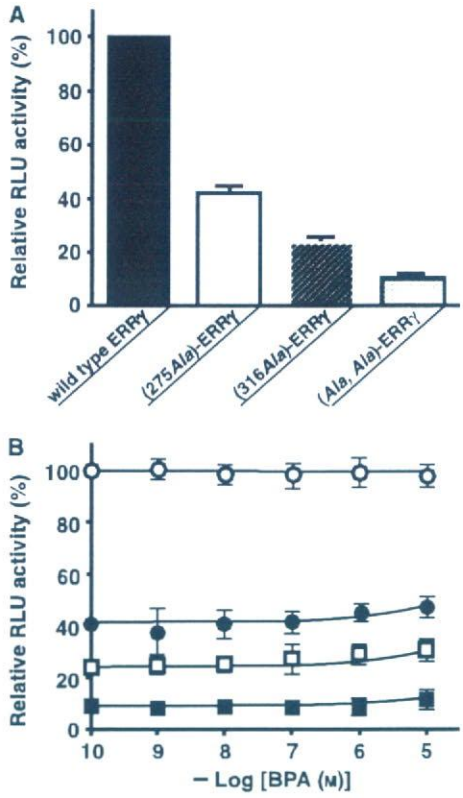

Fig. 6. Biological activity of the ERRγ and its site-directed mutant derivatives, by means of the luciferase-reporter gene assay. (A) The percentage relative potencies of a series of mutant receptors were measured against the basal constitutive activity of the wild-type ERRγ receptor (100%). An internal control that distinguishes the transcriptional level from variations in transfection efficiency was achieved by cotransfecting a second plasmid that constitutively expresses an activity that can be clearly differentiated from SEAP. (B) The effect of BPA on the basal constitutive activities of wild-type ERRγ (100%) and its mutant receptors. The graphs show the activity of wild-type ERRγ (○), (275*Ala*)-ERRγ (●), (316*Ala*)-ERRγ (□), and (*Ala, Ala*)-ERRγ (■) with 10^{-10} to 10^{-5} M BPA.

dose-dependent manner (Fig. 6B). For (275*Ala*)-ERRγ, 10 μM BPA increased the basal constitutive activity by 7%, reaching 49% of that of the wild-type ERRγ. For (316*Ala*)-ERRγ, 10 μM BPA also increased basal constitutive activity 7%, reaching 32% that of the wild-type ERRγ. This effect of BPA was found to be small (only approximately 3%) for (*Ala, Ala*)-ERRγ. These results clearly indicate that BPA functions to preserve the basal activity of ERRγ due to its strong binding, but that its binding to the mutant receptors is not sufficient to keep their conformation in a fully activated form. The Arg316 → Ala and Glu275 → Ala substitutions appear to damage intrinsically the activation conformation to a level that BPA is unable to rescue completely.

It was reported that 4-OHT deactivates ERRγ [12,24], diminishing the basal activity of ERRγ by up to 70–85% at a concentration of 10 μM (Fig. 7). BPA, on the other hand, showed no effect on the basal constitutive activity of ERRγ even at a concentration of 10 μM, completely preserving the high constitutive activity of ERRγ [12] (Figs 6 and 7). However, it should be noted that BPA reverses the inverse agonist activity of 4-OHT in a dose-dependent manner (Fig. 7). This effect of BPA has been acknowledged as an inverse antagonist activity on the constitutive activity of ERRγ [12]. Exactly the same receptor responses were observed for the (275*Ala*)-ERRγ mutant receptor (Fig. 7). It is noteworthy that the inverse agonist activity of 4-OHT and the inverse antagonist activity of BPA are observed for both (275*Ala*)-ERRγ and (316*Ala*)-ERRγ mutant receptors, and even for (*Ala, Ala*)-ERRγ.

Discussion

Differential capacity of Glu275 and Arg316 to interact with the ligand

In the present study, to inspect the structural elements of the ERRγ receptor in arresting BPA, we prepared 11 different analogue receptors with site-directed mutagenesis at positions 275 and 316. X-ray crystal structural analysis has suggested that the Glu275 and Arg316 residues each make a hydrogen bond with the phenol-hydroxyl group of BPA [20]. The present results clearly demonstrated that these residues are indeed involved in such hydrogen bonding interactions. Simultaneous mutation of these residues to Ala eliminated activity in binding to a BPA molecule, and individual mutations drastically reduced the activity. Because Ala lacks the characteristic side chains of Glu and Arg, the mutant receptors are devoid of the functional groups at the particular positions of 275 and 316. Thus, it becomes difficult for them to keep BPA in the ligand-binding pocket.

Interestingly, it became clear that Glu275 and Arg316 play roles in detaining BPA with different weights or levels of significance. The phenol-hydroxyl ↔Arg316 hydrogen bonding was found to play a major role, whereas the phenol-hydroxyl↔Glu275 hydrogen bonding plays a definite supporting role. In the saturation binding of [^{3}H]BPA, the extent of the decrease in the deactivation of the ERRγ receptor was much more drastic (by approximately 30-fold; Table 1) for the Arg316 → Ala substitution than that (approximately three-fold) for the Glu275 → Ala substitution,

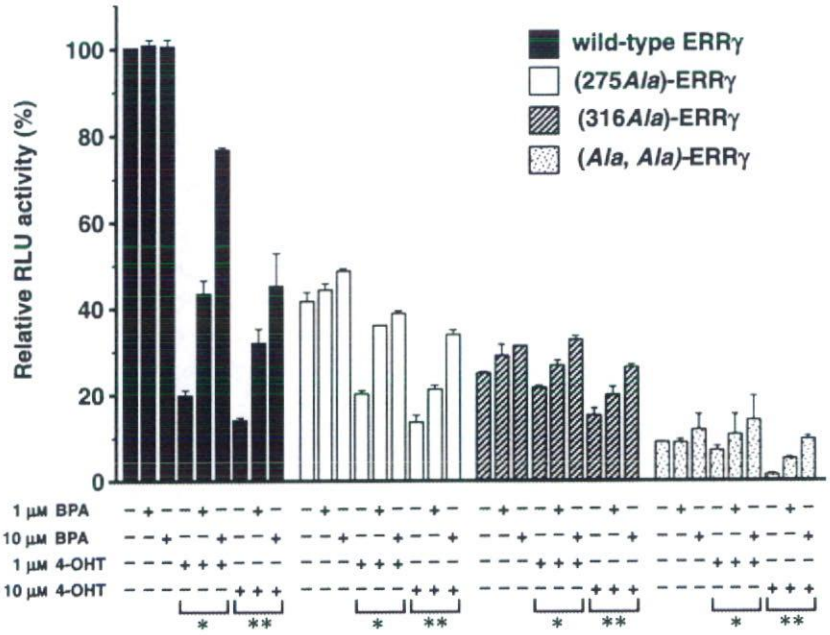

Fig. 7. Luciferase-reporter gene assays of BPA and 4-OHT for the ERRγ and its site-directed mutant derivatives. Assays were carried out to construct the concentration-dependent responses (1 and 10 μM) of BPA and 4-OHT in the luciferase-reporter gene assay. The basal constitutive activities of wild-type ERRγ (100%) and its mutant receptors were measured with no compounds. Normalization was achieved by simultaneous SEAP assays. The graphs show the basal constitutive activity, the activity of BPA (1 and 10 μM) for the basal constitutive activity, the inverse agonist activity of 4-OHT (1 and 10 μM) for the basal constitutive activity, and the inverse antagonist activity of BPA (1 and 10 μM) against the inverse agonist activity of 4-OHT (1 and 10 μM). The assay set marked with an asterisk shows the the inverse antagonist activity of BPA for 1 μM 4-OHT, and the other set marked by a double asterisk shows the the inverse antagonist activity of BPA for 10 μM 4-OHT. The receptors used are wild-type ERRγ, (275*Ala*)-ERRγ, (316*Ala*)-ERRγ, and (*Ala*, *Ala*)-ERRγ.

implying that Arg316 is much more important than Glu275 for [^{3}H]BPA binding.

It should be noted that the importance of the Arg-guanidino group was also demonstrated for the mutant receptor (*Arg*, *Glu*)-ERRγ, in which Arg and Glu are exchanged at the positions 275 and 316. (*Arg*, *Glu*)-ERRγ itself is still fairly potent for [^{3}H]BPA ($K_D \approx 60$ nM, approximately ten-fold larger than that of the parent ERRγ; Table 1). However, when the 275*Arg* → Ala substitution was given to this (*Arg*, *Glu*)-ERRγ mutant receptor, the resulting double-mutated receptor (*Ala*, *Glu*)-ERRγ became completely inactive for [^{3}H]BPA (Table 1). By contrast, another double-mutated receptor (*Arg*, *Ala*)-ERRγ, obtained by the 316*Glu* → Ala substitution, was found to be as active as the parent (*Arg*, *Glu*)-ERRγ (Table 1). The replacement of 316*Glu* with Ala had no effect on the binding ability of [^{3}H]BPA.

All these results clearly indicate the crucial role of Arg316 for the ERRγ receptor in ligand binding. This kind of structure–activity relationship between NRs and ligands has never been explored, and thus it is very important to seek an amino acid residue that is influential in, or definitive for, particular functions.

Evolutionary rationale for the major role of Arg316 in arresting the ligand

When the amino acid sequences of the LBD of all the NRs were aligned to that of ERRγ, it became noticeable that 26 receptors among the total 48 NRs [13] have Arg at the position corresponding to 316 (Fig. 8). In particular, all the members of Groups III, IV, and V NRs, consisting of nine, three, and two members, respectively, contain Arg at that particular position. There are seven Arg316-containing receptors in 19 Group I NRs and five in 12 Group II NRs. The fact that Arg316 is extremely highly conserved among NRs is remarkable because it constructs a part of the ligand-binding pocket inside each receptor. We reason that it must have been preserved in order to accept the similar structural elements of the ligands (e.g. the phenol-hydroxyl group) during the evolution of these diverse receptors.

On the other hand, Glu275 is conserved among only five NRs: ERs α and β, and ERRs α, β, and γ (Fig. 8). Although Glu possesses the carboxyl COOH group at the Cγ position, some other Arg316-containing NRs were found to have Gln at position 275. Instead of

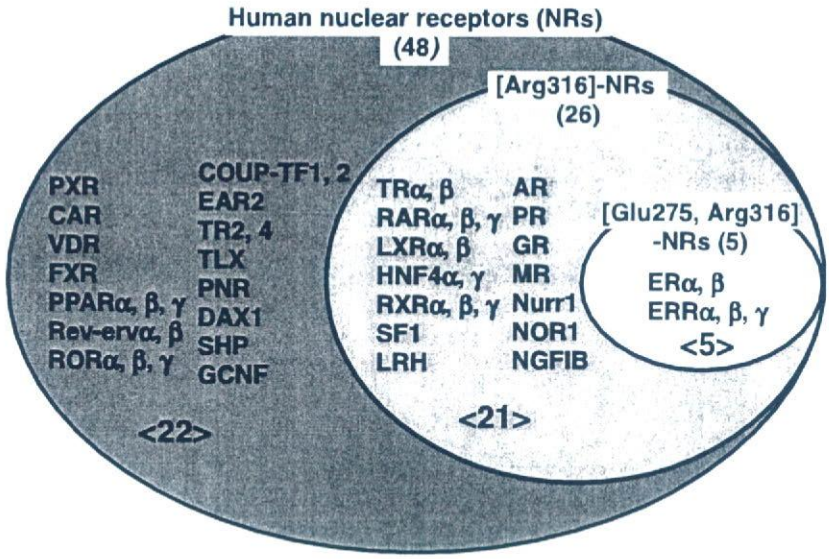

Fig. 8. Fractional grouping of the 48 human nuclear receptors according to residue variation at positions 316 and 275. Among 48 human nuclear receptors [13], the smallest is a group with five members whose nuclear receptors possess both Arg316 and Glu275, and the second group includes the 21 receptors containing Arg316.

COOH, Gln possesses the carboxyl amide $CONH_2$ group, which also retains both proton-donating and -accepting characters. However, as shown in the present study, Gln cannot necessarily replace the Glu275. It appears that (Glu275, Arg316)-containing NRs and (Gln275, Arg316)-containing NRs have different structural bases to receive each specific ligand.

Nine NRs contain the Gln275 and Arg316 residues simultaneously, and they belong to either Group II (five of 12) or Group III (four of nine) NRs. Other Arg316-containing NRs show a variety of amino acid residues at position 275: Ala ($n = 2$), Ser ($n = 5$), Thr ($n = 2$), and Cys ($n = 3$). When these residues including Gln are involved in the interaction with the ligand, they may be cooperative or collaborative with Arg316. All these details strongly suggest that Arg316 plays a principal role in selecting and binding the ligand for receptor activation. Of course, each individual NR should bind a specific ligand in a manner that differs from that by which other NRs bind their ligand, and thus the role of Arg316 must be different in some cases. Because the tasks played by Arg are varied and potent enough to cause the interaction with the ligand by means of electrostatic interaction, hydrogen bonding, and the so-called NH/π interaction, Arg316 may play the main role in arresting and keeping the ligand in the pocket.

Influence of residual mutation of ERRγ upon the basal constitutive activity

Compared to the high basal constitutive activity of the wild-type ERRγ receptor, the (275*Ala*)-ERRγ mutant receptor with the Glu275 → Ala substitution exhibited lessened, but still considerable basal activity (approximately 40% that of the wild-type) (Fig. 6). (275*Ala*)-ERRγ retains the Arg residue at position 316. However, mutant receptor Arg316 → Ala substitution showed very much weakened basal activity. (316*Ala*)-ERRγ exhibited basal constitutive activity, only approximately 20% that of the wild-type. Moreover (*Ala*, *Ala*)-ERRγ exhibited extremely weak basal activity. These data indicate that Arg316 is crucial in exhibiting biological activity as well as in ligand-binding.

In the case of the mutant receptor (275*Ala*)-ERRγ, with approximately 40% of the activity of wild-type ERRγ, 10 μM BPA only slightly enhanced activity (Figs 6 and 7). It appears to be difficult for BPA to completely occupy the ligand-binding pocket of (275*Ala*)-ERRγ. This is apparently because of the Glu275 → Ala substitution, and thus the slight increase in activity must be due to the ability of BPA to reconstruct an inactivated conformation into an activated one. BPA in the ligand-binding pocket of (275*Ala*)-ERRγ should hold H12 for the position in the active conformation. It is evident that such an effect of BPA is only partial, presumably because the binding of BPA to (275*Ala*)-ERRγ is not so stable. As for (316*Ala*)-ERRγ, this kind of reconstruction appears much more difficult.

For the inverse antagonist activity of BPA, the presence of an inverse agonist and its binding to the receptor is indispensable. 4-OHT exhibited reasonable receptor binding affinity for both the (275*Ala*)-ERRγ and (316*Ala*)-ERRγ receptors (Table 2) and, in the reporter gene assay, it showed definite inverse agonist activity for these mutant receptors, and even for

(*Ala*, *Ala*)-ERRγ (Fig. 7). BPA was found to clearly reverse the inverse agonist activity of 4-OHT in the wild-type ERRγ receptor and the mutant receptors, indicating that BPA displaces 4-OHT to convert to the activation conformation.

Conclusion

The present results reveal that ERRγ has residues (Gly275 and Arg316) to capture or arrest phenol compounds. Their individual substitutions revealed degrees of difference in activity reduction, indicating the major importance of phenol-hydroxyl↔Arg316 hydrogen bonding and the supportive role of phenol-hydroxyl↔Glu275 hydrogen bonding. The data obtained with characteristic mutations suggested that these hydrogen bonds are conducive to the recruitment of phenol compounds by ERRγ. The ERRγ receptor forms an appropriate structure presumably to adopt endogenous BPA-like ligand(s) that have yet to be identified.

Experimental procedures

Chemicals

BPA was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). 4-OHT was obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). [^{3}H]BPA (5 Ci·mmol^{-1}) was obtained from Moravek Biochemicals (Brea, CA, USA).

Plasmid construction and site-directed mutagenesis

A cDNA fragment encoding wild-type ERRγ-LBD (residues 222–458) was generated by PCR with specific primers 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) at the *Eco*RI and *Xho*I sites. Full-length wild-type ERRγ was also amplified from the human kidney cDNA library by PCR and cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) also at the *Eco*RI and *Xho*I 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 using pGEX-ERRγ-LBD or pcDNA3.1-ERRγ-Full as a template. The mutations were introduced by PCR mutagenesis in a two-step reaction [21]. The primers used were: 5′-ACTTGGCCGACCGA*xxx*TTGGTGGTTA-3′ (*xxx* = *gcg* for Glu275Ala, *cgg* for Glu275Arg, *gac* for Glu275Asp, and *ctg* for Glu275Leu); 5′-TCCTTGGTGTCGTATAC*xxx*TCTCTTTCA-3′ (*xxx* = *gcg* for Arg316 → Ala, *aag* for Arg316 → Lys, *ctg* for Arg316 → Leu, and *gag* for Arg316 → Glu). Each mutant LBD or full-length ERRγ was amplified and cloned into the vector pGEX-6p-1 or pcDNA3.1(+) at the *Eco*RI and *Xho*I sites. All PCR products were verified for their accuracy in the sequences. As an ERRE-luciferase construct, 3 × ERRE/pGL3 was used as described previously [12].

ERRγ-LBD protein expression

Two GST-fused receptor proteins (the wild-type and mutant GST-ERRγ-LBD) were expressed in *E. coli* BL21 as described previously [12]. The mixture was centrifuged, and the resulting pellet was sonicated in 2–20 mL of buffer (50 mM Tris/HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol). The receptor protein was purified by 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 phosphate buffered saline (NaCl/P$_i$) containing 0.2% (v/v) Triton X-100 and once with the same sonication buffer described above. Fusion protein was eluted with 1 M 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. The protein concentration was determined by the Bradford method [25].

Radioligand binding assays

Saturation binding

A saturation binding assay was conducted essentially as reported [26], by using [^{3}H]BPA. The reaction mixture was incubated overnight at 4 °C with the receptor proteins (GST-fused wild-type ERRγ-LBD or its mutants) in 100 μL 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^{-1} γ-globulins). The assay was performed with or without the addition of unlabeled BPA or 4-OHT (final concentration of 1×10^{-5} M) to quantify the specific and nonspecific binding. After incubation with 100 μL of 1% dextran-coated charcoal (Sigma) in NaCl/P$_i$ (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 nonspecific binding from the total binding, and the results were examined by Scatchard plot analysis. The assay was carried out at least in triplicate.