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Ligand-inducing Conformation Changes in the Estrogen Receptor C-Terminal Tail Moiety and Their Sensing by Polyclonal Antibodies

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Ligand binding to the estrogen receptor induces a conformation change. By using a polyclonal antibody to sense such a change, we have established the assay method to assess simultaneously the binding ability and hormonal activity of endocrine disruptors. In order to improve the immunoreactivity, we prepared four varieties of antibodies in this study. It was revealed that the ability of antibody to sense the conformation change is related to the structural characteristics of each antigen peptide.

Keywords: antigen peptide, conformation change, endocrine disruptors, estrogen receptor, polyclonal antibody.

Introduction

The estrogen receptor (ER) is a member of the nuclear receptor family which functions as a transactivation factor. The conformations of the ligand-bound (holo-ER) and ligand-free (apo-ER) forms of the ER are intrinsically different from each other [1]. This is due mainly to the change in positioning of the amphiphilic α-helix numbered as 12 (H12) present in the receptor C-terminal portion. This ligand-induced conformation change of the receptor is essential to bind the coactivator protein [2]. Antibodies provide a feasible tool to differentiate between these conformations, provided they could recognize specifically and selectively either the apo or holo conformation. In fact, an anti-ER H12 antibody was already found to discriminate apo-ER and holo-ER. In addition, this antibody was found to quantify the amount of ligand-bound and ligand-free ERs [3]. When compared with H12 *per se*, ER possesses a C-terminal tail including H12 which is about four times longer. It is highly likely that this tail moiety would also be involved in some conformation change. Thus, the purpose of the present study is to test the structural availability of this tail region as an antigen.

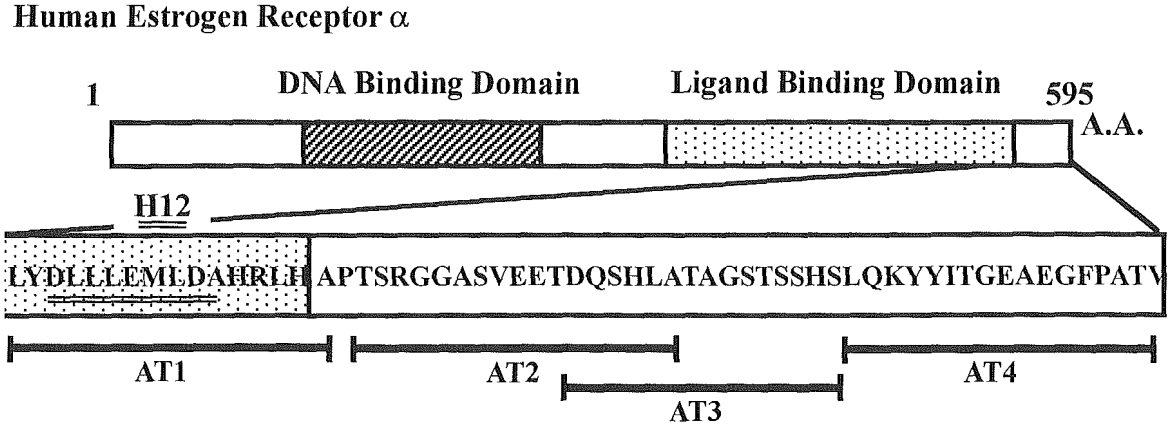

Figure 1. Design of antigen peptides for preparation of conformation-sensing antibody.

Results and Discussion

The C-terminal tail of ER was segmented into four peptide fragments (Fig. 1). The fragment AT1 contains H12. These peptides were synthesized by the Fmoc-based solid phase method. To conjugate to a carrier protein Keyhole Limpet Hemocyanin (KLH), Cys was incorporated into these peptides at the N-terminus. Peptides were liberated from the resin by treatment with Reagent K and purified by gel filtration (Sephadex G-25, 1.8 x 72 cm) followed by preparative reversed-phase high performance liquid chromatography (RP-HPLC) (Lichrospher RP-18 (e), 25 x 250 mm, 5 μm). The mass spectra of peptides were measured to verify their purity on a mass spectrometry Voyager™ DE-PRO with the method of matrix assisted laser desorption ionization time-of-flight (MALDI-TOF).

The peptides conjugated to KLH were injected into a rabbit, respectively. About three months later from the first immunization, blood was collected. The serum was purified successively by KLH immunoprecipitation, affinity chromatography with antigen-linked agarose gel, and then with a protein A-linked agarose gel. The specificity of antibody was analyzed by the enzyme-linked immunosorbent assay (ELISA) method. Competitive ELISA was employed to evaluate the ability of antibody to bind to apo-ER and/or holo-ER. The production of antibody was checked by preparative ELISA using ER and the antigen peptide. In this titer checking it was found that the serum contains enough amount of antibodies sensitive to both the receptor and peptide. As shown in Fig. 2, the serum obtained from the immunization by AT4 peptide interacted with the antigen peptide and the receptor ER almost equally well. Similar results were obtained for AT2 and AT3 peptides.

When the relative immunoactivity of the antibody was estimated under the certain concentrations of ER (10^{-12} - 10^{-6} M), a dose-dependent reduction was observed against the antigen peptide (10^{-12} - 10^{-11} mol) coated on the plate (Fig. 3). This assay was carried out under the presence of 10^{-5} M 17β-estradiol (E2), and the results were depicted in the same figure. As shown in Fig. 3A, a dose-dependent curve became much more gentle, making a certain deviation between the curves. This deviation (about 30%) corresponds to the sensing based on the conformation change. Thus, it is concluded that anti-AT1 exhibits a high ability to distinguish between holo-ER and apo-ER (Fig. 3). A similar result was obtained for anti-AT3 antibody, although the extent of deviation is much smaller than for anti-AT1 antibody. For anti-AT4

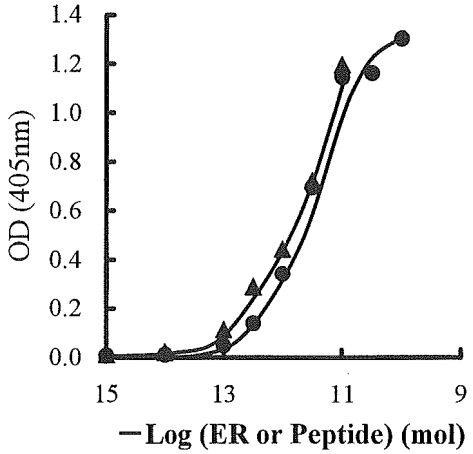

Figure 2. Indirect ELISA using anti-AT4 antibody for titer checking against recognition to antigen peptide (●) and ER (▲). The antibody used is the preparation purified by affinity chromatography with a protein A-linked agarose gel.

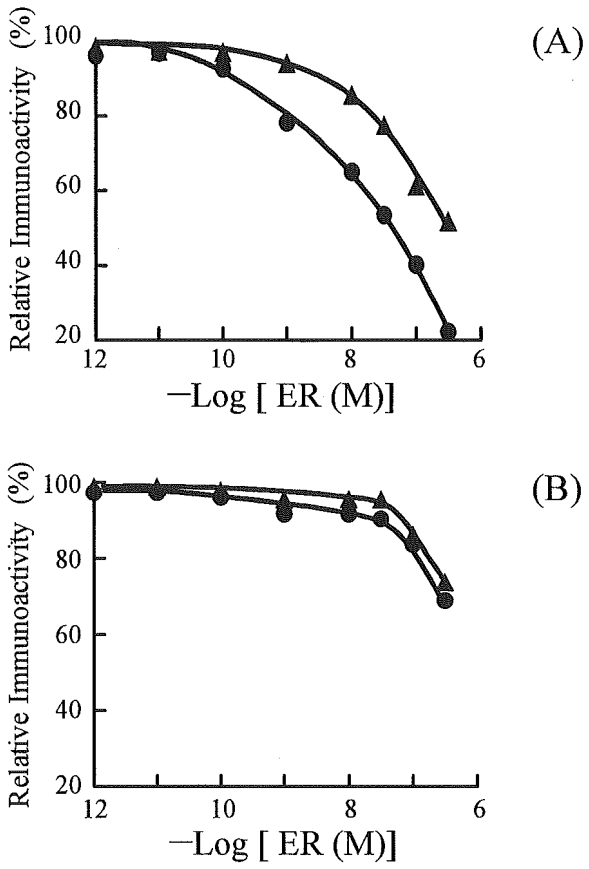

Figure 3. Immunoresponse of anti-AT1 (A) and anti-AT4 (B) antibodies against apo-ER (●) and holo-ER (▲). The antibodies used are the preparation purified by affinity chromatography with a protein A-linked agarose gel. Holo-ER was prepared by treatment with 10 μM 17β-estradiol.

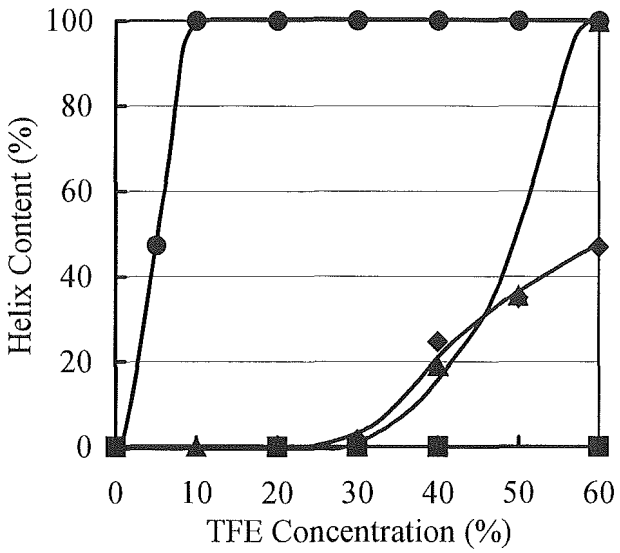

Figure 4. The helical characteristics of each peptide; AT1(●), AT2(■), AT3(▲),and AT4(◆)

antibody, almost no deviation was observed (Fig. 3B). A similar result was shown by anti-AT2 antibody (data not shown).

The CD spectra were masured on a J-725 Spectropolarimeter (Jasco), and the % contents of the secondary structures were calculated by SSE-338W protein secondary structure analysis program (Jasco). It was found that AT1 peptide is easy to adopt an α-helix structure. AT1 exhibited an extremely high content of α-helix (100% even in 10% TFE). AT3 peptide was found to adopt also a 100% α-helix structure, but only in 60% TFE. Almost no helical content was observed for AT2 peptide and a very low for AT4 peptide. These results indicated that the ability of antibody to sense the conformation change is well-related to the structural characteristics of each peptide fragment to adopt an α-helical conformation.

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α-Helix Peptides for Bio-Panning in the Phage Display Method to Obtain the Antibodies Specific for Conformation Change in Nuclear Receptors

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Ligand binding to the nuclear receptors (NRs) induces a conformation change. By using a polyclonal antibody to sense such a change, we have established the novel assay procedure to assess simultaneously the activities corresponding to the binding activity and hormonal activity of endocrine disruptors. In order to expand this method to 48 NRs, we attempted to prepare monoclonal antibodies (mAbs) by using phage-display system, and succeeded in the isolation of such mAbs from the Tomlinson I+J library.

Keywords: phage display, bio-panning, antigen peptide, conformation change, estrogen receptor, glucocorticoid receptor.

Introduction

A risk apprehension of endocrine disruptors for the sex hormone receptors has been extensively acknowledged for all of forty-eight human nuclear receptors (NRs) in these several years. It is thus a keen requisite to evaluate comprehensively the chemicals for each NR. We have recently established a novel assay procedure designated as 'conformation-sensing assay', which can estimate simultaneously the activities corresponding to the receptor binding activity and hormonal activity of the chemicals. This method is based on the quantification of a ligand-induced conformation change around the C-terminal domain of NRs, being due mainly to the change in positioning of the amphiphilic α-helix numbered as 12 (H12). The conformation change of H12 is a common feature of most of NRs, and is essential for recruit of coactivator protein [1].

We have recently demonstrated that a polyclonal antibody specific for H12 is able to sense such a conformation change, for example, for the estrogen receptor (ER) and glucocorticoid receptor (GR). A key material for successful conformation-sensing assay is an efficient antibody. In order to expand the methodology to all other NRs, we have attempted to obtain monoclonal antibodies (mAbs) by using phage-display system. This system has a great advantage that human single chain antibodies (scFvs) can be generated without immunization to animals [2]. In this study, to prepare such mAbs, Tomlinson I+J library was subject

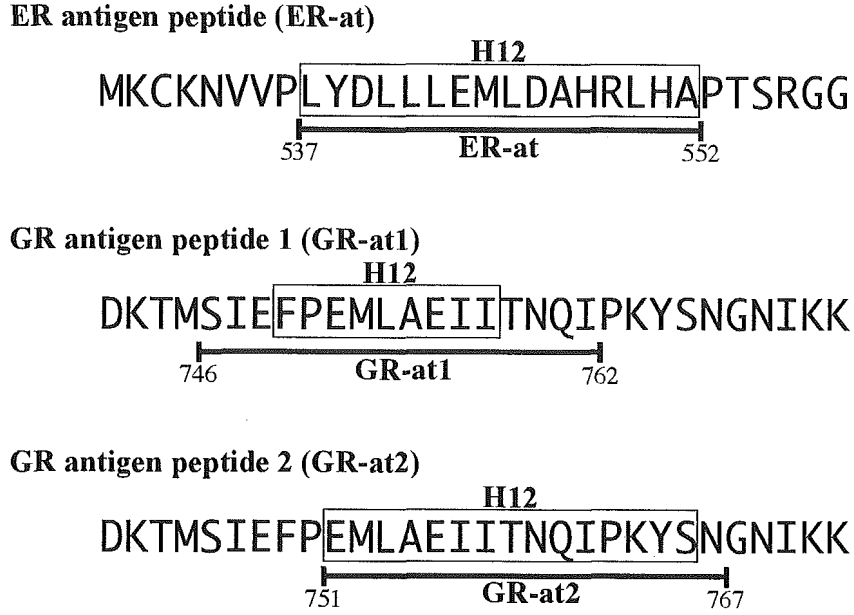

Fig. 1. The amino acid sequence of antigen peptides for bio-panning. Bars truncated indicate the fragment peptides (ER-at, GR-at1, GR-at2) used for preparation of protein KLH-linked antigen. Fragments in the box show the H12 α-helix portion found in each X-ray-clarified 3D-structures.

to bio-panning usingimmobilized antigen peptide containing H12.

Results and Discussion

In order to screen and select the phages desired, antigen peptides containing H12 peptides were synthesized by the Fmoc-based solid phase method. As shown in Fig. 1, two different peptide fragments were chosen as antigens, since GR-H12 was found to adopt α-helix differently against antagonist and agonist (Fig. 1) [3, 4]. To conjugate with a carrier protein keyhole limpet hemocyanin (KLH) or bovine thyroglobulin (BthG), Cys was attached at the N-terminus. Peptides were liberated from the resin by treatment with Reagent K. After gel filtration (Sephadex G-10), peptides were purified by reversed-phase HPLC (LiChrospher 100 RP-18 e (5μm) 4× 250 nm). The mass spectra of peptides were measured to verify their purity on a Voyager™ DE-PRO with the method of MALDI-TOF.

Tomlinson I+J library was kindly provided by Medical Research Council (MRC) (Cambridge, UK). The bio-panning for selection of phages that display scFv specific to antigens was performed repeatedly three times on an immuno-tube coated with purified antigen peptide. ER antigen peptide (ER-at) was immobilized in a 10% TFE/PBS (pH 7.2) solution, whereas GR antigen peptides GR-at1 and GR-at2 were immobilized indirectly as GR-at1 conjugated with KLH and GR-at2 conjugated with BthG. After three-round pannings, phage clones were screened for specific binding to the receptors by ELISA. As the receptors for screening, the ligand-binding domains (LBD) of ER and GR were utilized.

Although no clones specific to ER-LBD were isolated, a number of clones were found to recognize the antigen ER-at (Fig. 2). This result suggests that ER-at lost the appropriate secondary structure owing to directly adsorption onto immuno-tube. For GR, three clones specific to GR-LBD were found and isolated. Sequence analysis of these clones evidenced that two of them (6A and 11B) are

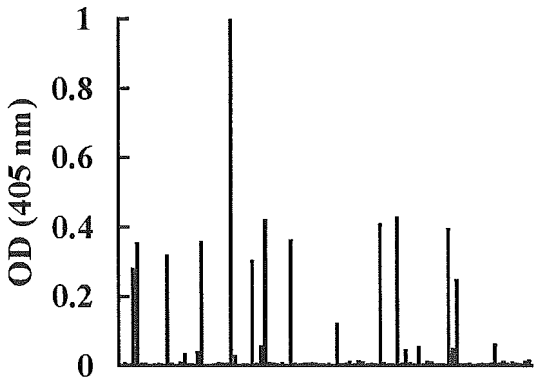

Fig. 2. Screening phage clones in ELISA to evaluate the specific binding to ER-at. The assay was carried out in 10% TFE. A number of clones were found to recognize ER-at, but none of them did bind to ER-LBD.

identical and do 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.

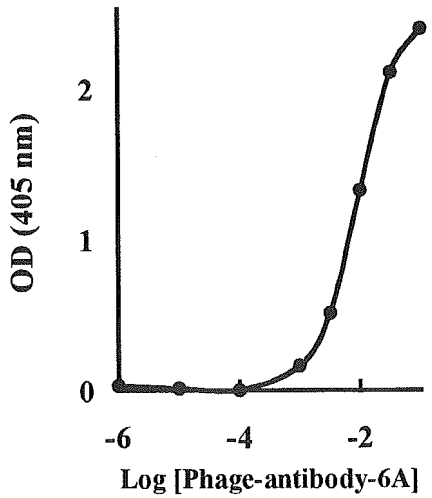

Fig. 3. GR-at1 binding curve for phage-antibody-6A by ELISA. GR-at1 was immobilized at 2.5 μg/ml. GR-at 2 binding curve was depicted almost as above.

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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].

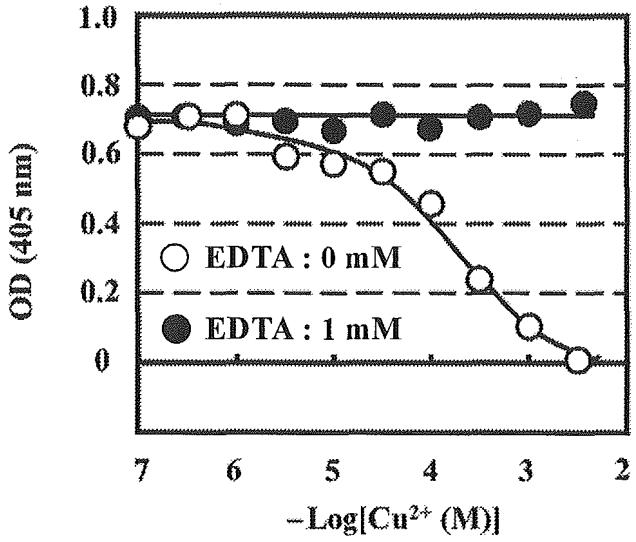

Fig. 1. Binding effect of Cu^{2+} to octarepeat peptide and recovery of the immunoresponse by addition of EDTA.

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 on 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 glucose regulation, 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 neurotropic functions. In the present study, we attempted to

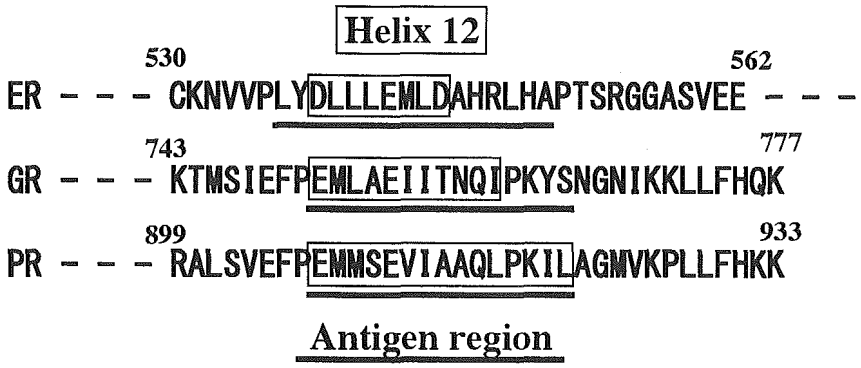

Fig. 1. Peptide fragments selected for preparation of the antigen to produce a conformation-sensing antibody. Cysteine was attached at the N-terminus of antigen peptides.

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 antigen peptides (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 receptor 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 onto 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 of chemicals were evaluated for GR and PR.

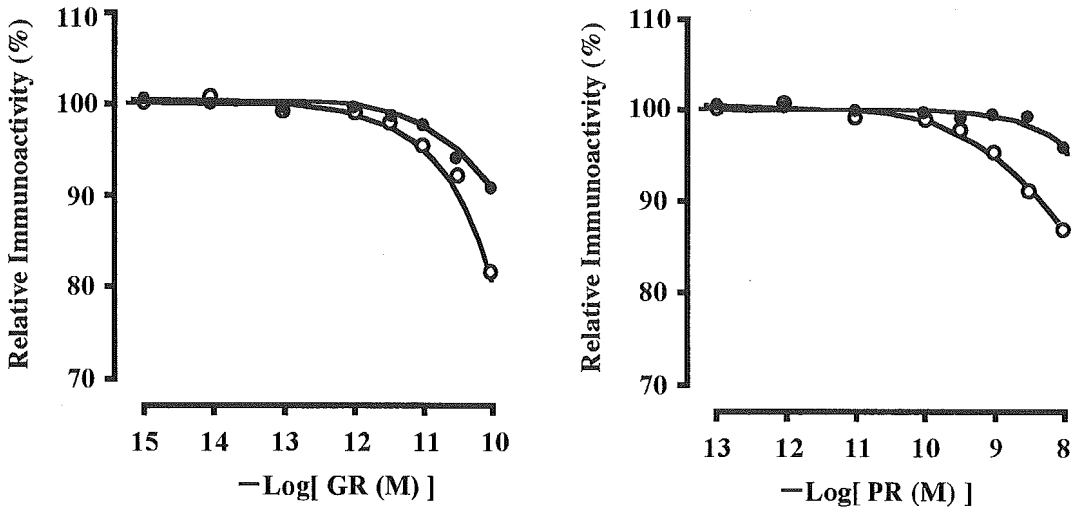

Fig. 2. Immunoresponses of anti-GR (A) and anti-PR (B) antibodies against apo-form (○) and holo-form (●) of each nuclear receptor. Holo-form was constructed with 10 μM deamethasone (A) and progesterone (B).

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 but EC_{50} value was not able to determine because of the low affinity.

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 but EC_{50} value was not able to determine because of the low affinity.

As shown in Table 1, cortisol, a natural agonist of GR, exhibited considerably weak activity (EC_{50} = 860 nM) as compared to that of Dex (32 nM). Although the result reflects the result of the receptor binding assay [1], this 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 discriminates distinctly 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 conformations of PR with agonists and antagonists.

It is known that PR ligands have bind also 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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