Expression of the Ligand-Binding Domain-Containing Region of Retinoic Acid Receptors α , β and γ in *Escherichia coli* and Evaluation of Ligand-Binding Selectivity

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The complete molecule or the ligand-binding domain-containing region of each of the three subtypes of human retinoic acid receptors (hRAR α , hRAR β and hRAR γ) was expressed in *Escherichia coli*. The expressed recombinant RARs (rRARs: rRAR α /E, rRAR β /E and rRAR γ) showed nearly the same magnitude of binding affinity toward [³H]retinoic acid (RA) as hRARs extracted from human cells (K_d values: $6.0 \times 10^9 \text{ M}^{-1}$ for rRAR α /E and $2.7 \times 10^{10} \text{ M}^{-1}$ for both rRAR β /E and rRAR γ). Therefore, the ligand-binding selectivity of the rRARs toward RA and synthetic retinoids (4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)benzoic acid (Am80), (E)-4-[3-(3,5-di-*tert*-butylphenyl)-3-oxo-1-propenyl]benzoic acid (Ch55)) was examined. Am80 bound rRAR α /E preferentially and showed no binding activity toward rRAR γ , which is consistent with the case of hRAR γ . Ch55 bound all three subtypes of rRARs, preferentially rRAR β /E. These results suggest that the intrinsic nature of the binding of each retinoid can be investigated by usage of the rRARs. However, rRARs show quantitatively different ligand-selectivity from that of hRARs: RA showed higher binding affinity toward rRARs than both Am80 and Ch55, but Ch55 binds all three subtypes of hRARs stronger than RA and Am80, which binds hRAR β stronger than RA.

Keywords retinoic acid receptor (RAR); retinoic acid; retinobenzoic acid; 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)benzoic acid (Am80); (E)-4-[3-(3,5-di-*tert*-butylphenyl)-3-oxo-1-propenyl]benzoic acid (Ch55); *Escherichia coli*

Retinoic acid (RA) plays fundamental roles in normal cell growth, cell differentiation and embryonic development in vertebrates.^{1,2)} Its various biological activities are mediated by its nuclear receptors (RARs).³⁾ RARs bind RA response elements which exist in the 5'-flanking region of RA-responsive genes and directly regulate the transcription of these genes in a ligand-binding-dependent fashion. Three subtypes of human RARs (hRAR α , hRAR β and hRAR γ) have been cloned, and are classed as members of the steroid/thyroid nuclear receptor superfamily.⁴⁻⁹⁾ RARs can be divided into six functional domains (A–F domains). The DNA-binding domain (C domain) and ligand-binding domain (E domain), each of which is highly conservative in amino acid sequence throughout the three subtypes, were shown to function independently.¹⁰⁻¹²⁾

On the other hand, a number of RA analogues have been developed in many laboratories, and their biological activities have been examined. These compounds, which elicit biological effects of RA, are referred to as "retinoids," regardless of their structural differences from RA.¹³⁾ Synthetic retinoids are expected to be useful therapeutic agents against proliferative dermatological diseases, leukemia and some types of tumors. Furthermore, these retinoids can be useful as tools to elucidate the molecular mechanisms of retinoidal actions. To understand the actions of the retinoids at the molecular level, characterization of their binding properties to the individual RAR subtypes is necessary.

In our previous studies, the existence of hRAR α and hRAR β in the nucleus of HL-60 cells was demonstrated by using our synthetic retinoids, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)benzoic acid (Am80) and (E)-4-[3-(3,5-di-*tert*-butylphenyl)-3-oxo-1-propenyl]benzoic acid (Ch55) (Fig. 1).^{14,15)} These two compounds, which are classed as "retinobenzoic acids," a

general term for a group of structurally novel retinoids developed in our laboratory, have potent retinoidal activities and bind RARs with high specificity (they do not bind efficiently to cellular retinoic acid-binding proteins (CRABPs)).^{16,17)} The RAR-subtype-binding selectivity of Am80 and Ch55 has been analyzed: *i.e.*, (i) Am80 binds RAR α and RAR β strongly, but it lacks binding affinity toward RAR γ , and (ii) Ch55 binds all three RAR subtypes with higher affinity than either RA or Am80.^{18,19)} In these previous studies, hRARs were prepared from the human cell line HL-60 by HPLC separation or from RAR-transfected COS-1 cells with a transient expression vector system. However, these methods for preparation of hRARs are complicated, because eukaryotic cell lines and tissues generally express multiple RAR subtypes and their contents are extremely low (a few thousand molecules per cell).^{9,18)} Production of recombinant RARs in *Escherichia coli* (*E. coli*) by genetic engineering techniques should allow us to prepare large amounts of individual RAR subtypes with ease and make detailed analysis of RA–RAR

Fig. 1. Structures of Retinoids Used in This Report

complexes using the physico-chemical methods (CD, NMR, X-ray) available.

In this article, we report the successful overexpression of recombinant RARs derived from the three subtypes of hRARs in *E. coli* using prokaryotic expression vector systems. Further, the ligand-binding properties of these recombinant RARs (rRARs) were examined.

Materials and Methods

Materials Am80 and Ch55 were synthesized as described previously.^{14,15} [³H]All-(E)-RA (51 Ci/mmol) was purchased from NEN. [³H]Am80 (65 Ci/mmol) was prepared at Amersham. Plasmids containing human RAR cDNA, pSG5-RAR α 0, -RAR β 0 and -RAR γ 0, were kindly provided by Prof. P. Chambon (INSERM, Strasbourg, France).^{4,7,9} Oligonucleotides were synthesized with an Applied biosystems model 391 DNA synthesizer and purified by reversed-phase HPLC. Rabbit polyclonal antibody specific for the E domain of RARs was a gift from Dr. M. Sato (The Tokyo Metropolitan Institute of Medical Science). This antibody was directed against a synthetic peptide coding a common

sequence in the E domains of the three subtypes. Expression vectors pKK223-3 and pET-3d are commercially available.

Construction of the Expression Plasmids Expression vector pKK223-3 (Pharmacia) was used for the expression of the ligand-binding domain of RAR α . pKK223-3 contains the *tac* promoter. pSG5-RAR α 0 was digested with *Sac*I and *Bam*HI. The *Sac*I-*Bam*HI fragment corresponding to the E and F domains of RAR α was ligated into the *Eco*RI and *Bam*HI sites of pKK223-3 by including synthetic *Eco*RI-*Sac*I adaptor in the ligation mixture to give pKK223-3-RAR α /E (Fig. 2a). The nucleotide sequence around the initiation codon is GAATTCTTATGGAGCTCATTGAGA.

For the expression of RAR β and RAR γ , expression vector pET-3d was used.²⁰ The vector pET-3d contains the promoter region of gene ϕ 10 from bacteriophage T7 and a unique *Nco*I site at the initiating ATG. This plasmid provides a strong expression system in combination with T7 RNA polymerase-inducible bacterial strains. The cDNA sequence of RAR β corresponding to the D, E and F domains was PCR-amplified. A *Xho*I site and a *Bam*HI site flanking the 5' and 3' ends, respectively, were added for cloning into pET-3d. A *Xho*I site was created in pET-3d by digestion with *Nco*I and *Bam*HI and by inserting a synthetic *Nco*I-*Xho*I-*Bam*HI adaptor. Subsequently, the PCR-amplified fragment was cloned into the *Xho*I and *Bam*HI sites to give pET-3d-RAR β /E (Fig.

Fig. 2. Construction of rRAR Subtype-Expression Plasmids

(a) pKK223-3-RAR α /E, (b) pET-3d-RAR β /E and (c) pET-3d-RAR γ . The boxes represent open reading frames of each hRAR subtype and black-hatched regions correspond to the ligand-binding domain (E domain) cloned into expression vectors. P_{tac} represents *tac* promoter. P₁₀ and T₁₀ represent, respectively, the promoter and the terminator derived from bacteriophage T7. (d) Schematic representation of rRARs proteins produced in *E. coli*. The amino acid numbers in each hRAR subtype according to references 7, 9 are shown on the boxes.

2b). The nucleotide sequence around the initiation codon of the resulting plasmid is CCATGGCTAGCTCAACTCGAGACAAAGAAT.

For the expression of RAR γ , the *Nco*I–*Bam*HI fragment coding full-length RAR γ from the initiating ATG was cleaved out from pSG5-RAR γ 0 and ligated into the *Nco*I site and *Bam*HI site of pET-3d to give pET-3d–RAR γ 0 (Fig. 2c).

Expression and Extraction of Recombinant RARs pKK223-3–RAR α /E was transfected into the *E. coli* strain JM109. pET-3d–RAR β /E or –RAR γ 0 was transfected into the strain BL21(DE3)pLysS. These bacteria carrying RAR expression plasmids were precultured in LB or SOB media with ampicillin (50 μ g/ml) and optional chloramphenicol (25 μ g/ml, only for BL21(DE3)pLysS). The overnight preculture was diluted 1:100 into M9 medium with 0.2% casamino acids, 0.2% glucose and ampicillin, and was grown at 37°C. After 3–4 h (A_{600} =0.8–1.0), isopropyl- β -D-thiogalactoside (IPTG) was added to 0.4 mM to induce RAR expression. After an additional 2 h, cells were harvested by centrifugation. The cells were resuspended in an extraction buffer (50 mM Tris–HCl, pH 8.0, 4°C, 50 mM NaCl, 10% glycerol, 1 mM 2-mercaptoethanol (2-ME), 1 mM α -phenylmethanesulfonyl fluoride (PMSF)) and lysed by sonication on ice. DNaseI (10 U/ml) and 10 mM MgCl₂ were added to digest DNA, and the mixture was incubated on ice for 30 min. The cell debris was removed by ultracentrifugation at 100000 $\times$ g, 4°C for 1 h. The supernatant was used for retinoid-binding assay or stored at –20°C.

Retinoid-Binding Assay The retinoid-binding activity of rRARs was estimated by the nitrocellulose filter binding assay method as described previously, with some modifications.¹⁷⁾ In brief and typically, the bacterial extract was diluted with a binding assay buffer (20 mM Tris–HCl, pH 8.0, 4°C, 0.3 M NaCl) and incubated with 4 nM [³H]RA or [³H]Am80 in the presence or absence of an unlabeled competitor (Am80, Ch55 and RA) at 4°C for 10–16 h. The incubation mixture was absorbed by suction onto a nitrocellulose membrane. The membrane was washed three times with an incubation buffer and then with 25% ethanol in distilled water. Radioactivity that remained on the membrane was measured in Atomlight (NEN) by using a liquid scintillation counter. Retinoid solution was prepared as a 0.2 M dimethyl sulfoxide (DMSO) stock solution. Final DMSO concentration in the incubation mixture was 2%. For Scatchard analysis, bacterial extracts were incubated with 5–200 nM [³H]RA.

Other Techniques Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 10–20% gradient gel) was performed according to Laemmli,²¹⁾ and proteins were stained with Coomassie Blue. Western blotting was performed with a semi-dry blotting system (ATTO) and blotted RARs were detected by using an antibody specific for the E domain of RARs, with a Blotting detection kit (Amersham).

Results

Expression of Recombinant Human RARs in *E. coli* In our expression study, truncated RARs which contain only the ligand-binding domain (E domain) without the DNA-binding domain (C domain) were preferred, because there seemed no necessity to include the DNA-binding domain to analyze the ligand-binding properties of RARs: the functions of these two domains have been shown to be separable.^{10–12)}

For the construction of RAR expression plasmids, prokaryotic expression vectors pKK223-3 and pET-3d were used. cDNA fragments coding the ligand-binding domain of hRAR α and hRAR β were respectively cloned into pKK223-3 and pET-3d as described in Materials and Methods to give pKK223-3–RAR α /E and pET-3d–RAR β /E, respectively (Fig. 2a, b). Poor expression of truncated hRAR γ , for whose truncation the *Sac*I site at the same position as in hRAR α was used, in both systems led us to use full-length cDNA of hRAR γ , which was cloned into pET-3d to give pET-3d–RAR γ 0 (Fig. 2c). Figure 2d shows the translated region of rRARs produced in the bacterial cells.

Bacterial cells (JM109 or BL21(DE3)pLysS) transfected with the resulting plasmids (pKK223-3–RAR α /E, pET-3d–RAR β /E or pET-3d–RAR γ 0) were grown at 37°C. Production of recombinant proteins was induced by the addition of 0.4 mM IPTG. After 2–3 h of induction, whole cells, soluble fraction or insoluble fraction were subjected to SDS–PAGE analysis. High-level expression of truncated RAR α and RAR β (rRAR α /E and rRAR β /E) was observed (Fig. 3a, lanes 2 and 4), although the majority of rRAR α /E and rRAR β /E was pelleted as inclusion bodies or aggregates upon cell lysis by sonication and a small portion was recovered in the soluble fractions (Fig. 3c). The estimated molecular masses of rRAR α /E and rRAR β /E on Coomassie-stained SDS gels coincided with the predicted

Fig. 3. SDS–PAGE Analysis and Immunoblotting of Recombinant RARs Expressed in *E. coli*

(a) Coomassie-stained SDS–PAGE, on 10–20% gradient gel, of total crude extracts from bacterial cells. JM109 harboring pKK223-3–RAR α /E (lanes 1 and 2) or BL21(DE3)pLysS harboring pET-3d–RAR β /E (lanes 3 and 4) or BL21(DE3)pLysS harboring pET-3d–RAR γ 0 (lanes 5 and 6) was grown at 37°C without IPTG (lanes 1, 3 and 5) or for 2 h with 0.4 mM IPTG (lanes 2, 4 and 6). rRAR α /E and rRAR β /E are indicated by arrows. (b) Immunoblotting analysis of rRAR α /E (lane 1) or rRAR β /E (lane 2) in total bacterial extracts. (c) Coomassie staining of total extracts (lanes 1 and 4) or soluble fractions (lanes 2 and 5) or insoluble fractions (lanes 3 and 6), prepared from JM109 expressing rRAR α /E (lanes 1–3) or BL21(DE3)pLysS expressing rRAR β /E (lanes 4–6).

values, 32 and 36 kDa, respectively. Both rRAR α /E and rRAR β /E were identified by Western analysis with E domain-specific antibody (Fig. 3b). The expression level of rRAR γ was extremely low (Fig. 3a, lane 6), so we could not identify a specific band of rRAR γ on the stained gel nor by the Western analysis system used in this report.

Next, the binding activities of [3 H]RA toward each crude cell extract was examined. The binding to [3 H]RA was detected only when the expression of rRARs was induced with IPTG (Fig. 4) and was subject to competition by unlabeled RA, but not retinol, retinal or estrogen, indicating that the [3 H]RA-binding activities are specific.

Fig. 4. Inducible Expression of Ligand-Binding Activity by the Addition of IPTG

Each bar represents the specific radioactivity detected by the incubation of the crude cell extract of each rRAR subtype with 4 nM [3 H]RA. Bacterial cells were cultured in the absence (—) or the presence (+) of 0.4 mM IPTG. Equal amounts of extracts were used in assays.

Specific binding activity of rRAR γ was also detected. (The binding assay system is extremely sensitive; it could detect a few fmol of active proteins, although their expression in *E. coli* was too low to detect on the stained SDS gel). These results indicate that RARs which retain retinoid-binding activity can be expressed in *E. coli* and brought us to the conclusion that active rRAR γ was also expressed in *E. coli*. Though the overexpressed rRARs (rRAR α /E and rRAR β /E) were predominantly expressed in the insoluble fractions (Fig. 3c), attempts to solubilize and renature rRARs in the pelleted insoluble fractions using denaturing agents were unsuccessful. In the following experiments, rRARs extracted in the crude soluble fraction were used.

Affinity of rRARs toward [3 H]RA Association constant (K_a) values of the rRARs toward [3 H]RA were estimated by Scatchard analysis. As shown in Fig. 5, K_a values were found to be $6.0 \times 10^9 \text{ M}^{-1}$ for rRAR α /E and $2.7 \times 10^{10} \text{ M}^{-1}$ for both rRAR β /E and rRAR γ . The values are in good accordance with those for hRAR subtypes extracted from eukaryotic cell lines,¹⁹⁾ suggesting that rRARs produced in *E. coli* would possess identical (or similar) ligand-binding characteristics to those of hRARs in eukaryotic cells.

Ligand-Binding Selectivity of rRAR Subtypes Previously we reported the binding characteristics of RA and two synthetic retinoids (Am80 and Ch55) toward hRAR subtypes prepared from eukaryotic cells.^{18,19)} In those studies we found that: (i) Ch55 possesses the strongest binding affinity toward all three hRAR subtypes among the three retinoids, and (ii) Am80 binds to hRAR α and hRAR β with comparable affinity to RA, but does not bind hRAR γ .

To examine whether rRARs possess the same (or similar)

Fig. 5. Scatchard Analysis of [3 H]RA-Binding to Each of the rRARs

(a) rRAR α /E; $K_a = 6.0 \times 10^9 \text{ M}^{-1}$, (b) rRAR β /E; $K_a = 2.7 \times 10^{10} \text{ M}^{-1}$, (c) rRAR γ ; $K_a = 2.7 \times 10^{10} \text{ M}^{-1}$. [3 H]RA (5–200 pM) was incubated with each of the rRARs in crude bacterial extracts. Each point was assayed in duplicate. The lines were fitted by the least-squares method.

Fig. 6. Competitive Binding Curves of Retinoids with rRARs

(a) rRAR α /E, (b) rRAR β /E, (c) rRAR γ . rRAR crude extracts were incubated with a fixed amount of [3 H]RA (4–8 nM) in the presence of unlabeled retinoids ($\blacktriangle$, RA; $\blacksquare$, Am80; $\circ$, Ch55) at 0.1 to 500-fold concentration. Here 100% indicates the amount of specifically bound [3 H]RA, calculated by the subtraction of nonspecifically bound [3 H]RA (in the presence of a 500-fold excess of unlabeled RA) from total bound [3 H]RA (in the absence of unlabeled retinoids).

TABLE I. Relative Binding Activity of RA, Am80 and Ch55 for Each rRAR Subtype

Compound	Relative binding to ^{a)}		
	α	β	γ
RA	100	450	450
Am80	16	3	<0.2
Ch55	100	450	18

a) The binding activity of RA with rRAR α /E is defined as 100.

ligand-binding characteristics as the corresponding hRARs, binding competition experiments were carried out using [³H]RA and unlabeled RA, Am80 and Ch55. The results are shown in Fig. 6. The affinity of Ch55 to rRAR α /E and rRAR β /E was almost the same as that of RA; not so high as expected from the results obtained by using hRARs.^{18,19)} Toward rRAR γ , its affinity was 25 times lower than that of RA. In the case of Am80, the extremely low affinity to rRAR γ (almost no binding) is consistent with that of hRAR γ produced in eukaryotic cells. However, it binds to rRAR β /E only weakly (150 times less than RA).

Table I summarizes the relative affinities of the three compounds to the rRARs calculated by taking the affinity of RA for rRAR α as 100. From the viewpoint of the subtype-selectivity of ligands, Am80 and Ch55 can be considered to be α -selective and β -selective retinoids, respectively, in this rRARs system. Such a clear-cut binding selectivity of rRARs is surprising, because the ligand-binding domains of the RARs share considerable identity.

Discussion

The ligand-binding domain-containing region or whole molecule of each of the three hRAR subtypes (rRAR α /E, rRAR β /E and rRAR γ) were produced in *E. coli* by using prokaryotic expression systems. Using these rRARs, retinoid-binding properties were studied. In the case of α and β subtypes, their DNA-binding domain (C domain) was omitted and only the ligand-binding domain (E domain and flanking region) was overexpressed efficiently, although the majority was expressed as insoluble and inactive proteins. Other approaches to express them as fusions to proteins from *E. coli* may improve the solubility of the rRARs. RAR γ was expressed in a full-length form. The poor expression of the full-length form of RAR γ and no expression of the truncated form of RAR γ are presumably due to their disadvantageous codon usage and mRNA sequences around the translation initiation sites or in the 3'-untranslated region for translation in *E. coli*.

Scatchard analysis showed that these rRARs extracted into soluble fraction possess almost the same affinity for RA as hRARs extracted from eukaryotic cells (K_a values; $6.0 \times 10^9 \text{ M}^{-1}$ for rRAR α /E, $2.7 \times 10^{10} \text{ M}^{-1}$ for rRAR β /E and rRAR γ). The K_a value for rRAR α /E corresponds to 0.17 nM as a dissociation constant (K_d) value, and this is in good accordance with the values for rRAR α expressed in *E. coli* reported by other investigators ($K_d = 0.67 \text{ nM}$, Keidel *et al.*; $K_d = 0.21 \text{ nM}$, Yang *et al.*).^{22,23)} On the other hand, Crettaz *et al.* reported the K_d value for the recombinant ligand-binding domain of RAR α as 6.2 nM, *i.e.* 10–36 times lower,²⁴⁾ and Keidel *et al.* concluded that the isolated

ligand-binding domain is not identical to that of the entire receptor.

We previously reported the K_a values for the binding to RA of hRAR α and hRAR β extracted from the human promyelocytic leukemia cell line HL-60 (hRAR α ; $K_a = 3.7 \times 10^{10} \text{ M}^{-1}$, hRAR β ; $K_a = 2.2 \times 10^{10} \text{ M}^{-1}$).¹⁹⁾ The K_a value for rRAR β /E is in good agreement with the value we have reported, although the K_a value for rRAR α /E is 6 times lower than that for hRAR α .

Am80 and Ch55 are synthetic retinoids developed in our laboratory and have more potent biological properties than those of RA in several bioassay systems (*e.g.* differentiation induction of HL-60).^{14,15)} In addition, Am80 was less toxic than RA in a clinical trial (K. Shudo, unpublished data). It might be possible to interpret the characteristic retinoidal activities of each retinoid molecule in terms of its differential binding properties to RAR subtypes. Therefore, the receptor-binding characteristics of each retinoid attracted our interest. Previously we examined the order of affinity of the three retinoids (RA, Am80 and Ch55) for hRAR α and rRAR β , which were extracted from HL-60 cells and separated by anion-exchange HPLC, by means of binding competition experiments. The studies demonstrated that Ch55 bound most strongly to both subtypes, and Am80 bound more strongly than RA to the β -subtype. The results, together with other results obtained later, may be summarized as follows: the order of binding affinity to hRAR α is Ch55 > RA > Am80, that to hRAR β is Ch55 > Am80 > RA, and that to hRAR γ is Ch55 > RA >> Am80 (no binding).¹⁹⁾ In this report similar studies were carried out using rRARs. The results showed a rather different order: rRAR α /E, Ch55 = RA > Am80; rRAR β /E, Ch55 = RA > Am80; rRAR γ , RA > Ch55 >> Am80 (no binding). Though the affinities of the three retinoids for the rRARs are quantitatively different from those for the hRARs, the intrinsic nature of the binding of the compounds could still be investigated by the usage of the rRARs: Am80 does not bind either hRAR γ or rRAR γ , while RA and Ch55 bind all of the hRARs and rRARs. Therefore, it could be concluded that the binding activity of the compounds to each hRAR subtype can be predicted by the usage of the corresponding rRARs, though the binding activity cannot be quantitatively estimated. The differences of quantitative estimation of binding activities between hRARs and rRARs suggests the possibilities that intrinsic binding activity exists in the E domain, although other domains may stabilize the receptor–ligand complex, or rRAR proteins may be folded incorrectly in part but sufficiently to bound ligands. Even though the behavior of rRARs is not identical with that of hRARs under given conditions, detailed studies using rRARs should be helpful to elucidate the characteristics of RARs *in vivo*.

Recently, the identification of RAR subtype-selective retinoids has been reported by some groups.^{24–26)} Such selective compounds are expected to be useful tools for biological studies and for the development of superior therapeutic agents. Table I shows that Am80 is essentially an α -selective retinoid and possesses almost no affinity for the γ -subtype in the recombinant RAR system. For further identification of selective agonists and/or antagonists, investigation using both receptor binding and biological

activity assay systems is essential. Binding assay using the recombinant RAR proteins reported here should provide valuable information.

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