Expression of human *all-trans*-retinoic acid receptor β and its ligand-binding domain in *Escherichia coli*

Maria BERGGREN SÖDERLUND,* Gunvor JOHANNESSON and Göran FEX
Department of Clinical Chemistry, University Hospital of Lund, S-221 85 Lund, Sweden

all-trans-Retinoic acid, one of the hormonally active derivatives of vitamin A, occurs physiologically in plasma at a concentration below 10 nmol/l. The methods currently used for its quantification are based on HPLC, need about 1 ml of serum, are relatively laborious and thus not well suited for mass analysis. The affinity and specificity of retinoic acid receptors for *all-trans*-retinoic acid encouraged us to express both the entire human retinoic acid receptor β (RAR-β) and two versions of its retinoic acid-binding domain in *Escherichia coli* in the hope that these recombinant proteins might be used as binders in a ligand-binding assay for *all-trans*-retinoic acid. The recombinant receptors, the whole receptor [RAR-β-(V7–Q448)], corresponding to domains A–F, and the ligand-binding domain [RAR-β-(E149–Q448)], corresponding to domains D–F, were expressed in the vector pET 3d/BL21(DE3) as inclusion bodies, solubilized with guanidinium chloride, renatured and purified by ion-exchange chromatography. RAR-β-(P193–Q448), corresponding to domains E–F, was expressed in the vector pET 3d/BL21(DE3)pLysS, and purified by reversed-phase chromatography. Under non-denaturing conditions, the expressed whole receptor [RAR-β-(V7–Q448)] and the D–F construct (RAR-β-(E149–Q448)] behaved chromatographically as monomeric proteins whereas the E–F construct [RAR-β-(P193–Q448)] had a strong tendency to aggregate. RAR-β-(V7–Q448) and RAR-β-(E149–Q448) had similar K_d values for *all-trans*-retinoic acid (1.4 and 0.6 nmol/l respectively) whereas RAR-β-(P193–Q448) bound *all-trans*-retinoic acid less avidly (K_d 9.6 nmol/l). 9-*cis*-Retinoic acid bound to RAR-β-(E149–Q448) and RAR-β-(V7–Q448) as avidly as *all-trans*-retinoic acid. Competition experiments showed weak or no binding of 4-oxo-*all-trans*-retinoic acid, 4-oxo-13-*cis*-retinoic acid, 13-*cis*-retinoic acid, acitretin and retinol by RAR-β-(E149–Q448).

INTRODUCTION

all-trans-Retinoic acid, one of the hormonally active derivatives of vitamin A, occurs in plasma and other body fluids at concentrations below 10 nmol/l [1–3]. The methods currently used for its quantification [3,4] are based on HPLC. They work well but consume about 1 ml of serum, depend on relatively expensive equipment, consume large volumes of solvents, are relatively laborious, expensive and not well suited for mass analysis. A binding assay for *all-trans*-retinoic acid using cellular retinoic acid-binding protein as a binder has been described [5] but seems not to be sufficiently sensitive.

Commercial ligand-binding assays for 1,25-dihydroxy-vitamin D based on the cellular vitamin D receptor work well [6]. It might therefore be possible to use one of the retinoic acid receptors in a similar way to quantify *all-trans*-retinoic acid. The affinities of the different retinoic acid receptors for *all-trans*-retinoic acid differ. Of the retinoic acid receptors tested so far [7], the β-receptor and the γ-receptor seem to have the highest affinities for *all-trans*-retinoic acid.

Recently, it was demonstrated that the recombinant ligand-binding domain of the retinoic acid receptor β (RAR-β) expressed in *Escherichia coli* retained some affinity for retinoic acid [8]. This encouraged us to express both the entire human RAR-β and two versions of its retinoic acid-binding domain in *E. coli*, in the hope that they might bind *all-trans*-retinoic acid with high enough affinity to be used in a ligand-binding assay for *all-trans*-retinoic acid.

In this paper the production and partial purification of the recombinant RAR-β and its ligand-binding domain are described, and their retinoid-binding profiles characterized.

MATERIALS AND METHODS

RAR-β cDNA was a gift from Dr. Anne Dejean, Paris, France. The expression vector pET-3d [9], antibodies to the N-terminal extension sequence in the expressed fusion protein, and host bacteria were obtained from Novagen (AMS Biotech, Stockholm, Sweden). [^{3}H]*all-trans*-Retinoic acid (specific radioactivity 50 mCi/μmol) was from Du Pont, NEN Research Products (Kista, Sweden). Unlabelled *all-trans*-, 9-*cis*- and 13-*cis*-retinoic acid, the 4-oxometabolites of *all-trans*- and 13-*cis*-retinoic acid (Ro 12-4824 and Ro 22-6595) and acitretin were gifts from Hoffman-LaRoche (Basel, Switzerland). Unlabelled retinol was from Sigma (St. Louis, MO, U.S.A.). All handling of retinoids was carried out in the dark or under yellow light. Guanidinium chloride (98%, w/w) was from Janssen Chimica (Beerse, Belgium), rifampicin and isopropyl thio-β-D-galactopyranoside (IPTG) were from Sigma, leupeptin was from Peninsula Laboratories (Belmont, CA, U.S.A.) and aprotinin was from Bayer Sverige (Gothenburg, Sweden).

Construction of the expression vector

Upstream oligonucleotide primers, with built-in restriction sites for *Bam*HI or *Nco*I (restriction site in bold type), corresponding

Abbreviations used: RAR-β, retinoic acid receptor β; IPTG, isopropyl thio-β-D-galactopyranoside; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

* To whom correspondence should be addressed.

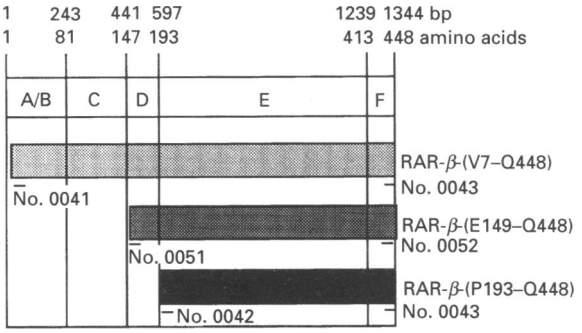

Figure 1 Three variants of recombinant RAR-β

The bars show the size of the expressed receptors. RAR-β-(V7–Q448) contains the amino acid residues Val^{7}–Gln^{448}, RAR-β-(E149–Q448) contains Glu^{149}–Gln^{448} and RAR-β-(P193–Q448) contains Pro^{193}–Gln^{448}. The numbers indicate the PCR primers used (see the text). The lettering refers to the terminology of the domains of the receptors of the steroid hormone superfamily [10]: A/B, N-terminal region; C, cysteine-rich DNA-binding region; D, hinge region; E, ligand-binding region; F, C-terminal region.

to the N-terminus of RAR-β (no. 0041: 5′-TTC AAT CGG ATC CTA GTT CTG TCA GTG AGT), the two different upstream primers of the ligand-binding domain of RAR-β (no. 0051: 5′-TT ACC ATG GAA TCT GTC AGG AAT GAC AGG AAC and no. 0042: 5′-TTC AAT CGG ATC CTA CCT TCA CTC TGC CAG) and the downstream primers at the C-terminus of RAR-β (no. 0043: 5′-TTC TTC GGA TCC TTA TTG CAC GAG TGG TGA and no. 0052: 5′-TTA CCA TGG TTA TTG CAC GAG TGG TGA CTG AC) (Figure 1) respectively were synthesized using an Applied Biosystems model 392 nucleic acid synthesizer. The oligonucleotides were used to amplify the corresponding portions of RAR-β from RAR-β cDNA, using 30 cycles of 1 min at 95 °C, 1 min at 43 °C and 1 min at 72 °C in a Perkin–Elmer thermal cycler. The amplified fragments were cleaved with *Bam*HI or *Nco*I and purified by agarose-gel electrophoresis.

The cleaved and purified fragments with *Bam*HI and *Nco*I ends were ligated into *Bam*HI- or *Nco*I-cleaved pET-3d and subcloned in HB101. The orientation of the insert in the pET-3d constructs was determined with PCR using oligonucleotide no. 0044 (5′-TAA TAC GAC TCA CTA TAG GG) (which corresponds to the sequence of the T7 promoter in pET-3d) as the upstream primer and no. 0043 (above) as the downstream primer. Colonies containing plasmid with the insert in the correct orientation were grown in Luria–Bertani medium containing ampicillin (50 mg/ml), and plasmids were isolated by standard procedures [11].

The plasmid constructs pET[RAR-β-(V7–Q448)] (whole RAR-β, domains A–F), pET[RAR-β-(E149–Q448)] and pET[RAR-β-(P193–Q448)] (the two versions D–F and E–F of the ligand-binding domain of RAR-β) (Figure 1) were then transfected into BL21(DE3) and BL21(DE3)pLysS respectively.

Expression of RAR-β-(V7–Q448), RAR-β-(E149–Q448) and RAR-β-(P193–Q448)

The bacteria containing the expression vectors for the different variants of RAR-β were plated on Luria–Bertani plates containing ampicillin (50 mg/ml). Colonies were picked and grown in Luria–Bertani medium containing ampicillin (150 mg/ml) overnight. The culture was diluted 1:100 and grown to A_{600} 0.6–1.0 [12]. At this stage cells were induced with IPTG (0.40 mM) for 3 h at 37 °C. Rifampicin (200 μg/ml) was added 30 min after induction with IPTG as it increased the yield of recombinant protein.

A small portion of the resulting bacterial cell suspensions was dissolved in sample preparation buffer and subjected to SDS/PAGE and Western blotting (not shown) [13–15], using antibodies to the N-terminal extension sequence from pET-3d or the synthetic RAR-β-peptide Arg^{155}–Ser^{162} (available at the laboratory) to demonstrate semiquantitatively the induction of the whole receptor, RAR-β-(V7–Q448) (approx. 55 kDa), and the two versions of its ligand-binding domains, RAR-β-(E149–Q448) and RAR-β-(P193–Q448) (approx. 36 and 33 kDa respectively) (see Figure 2). All three proteins were expressed mainly as inclusion bodies.

Purification and solubilization of the inclusion bodies and purification of the recombinant proteins

For some purposes (indicated in the text) a crude receptor extract was prepared by extracting the bacterial pellet from 100 ml of culture in 50 mmol/l Tris/HCl, pH 8.0, containing 0.1 % (v/v) Triton X-100 and 200 μg/ml lysozyme by freezing (−70 °C) and thawing, sonication till the solution lost its viscosity and centrifugation for 30 min at 17000 ***g***. The resulting supernatant was used after appropriate dilution. For most experiments cells from 100 ml of culture were collected by centrifugation and treated essentially as described by Lin et al. [16].

RAR-β-(E149–Q448) (domains D–F)

RAR-β-(E149–Q448) was purified in three steps by ion-exchange chromatography [Q-Sepharose fast flow column (Pharmacia, Uppsala, Sweden) at pH 8.0, Mono Q HR 10/10 column (Pharmacia) at pH 7.5 and Mono S HR 5/5 column (Pharmacia) at pH 6.6].

Before the extract was applied to an ion-exchange column, it was incubated with about 2 μCi of [^{3}H]*all-trans*-retinoic acid at room temperature in the dark for 10 min. The columns were eluted with gradients from 0 to 1 mol/l NaCl. The RAR-β-(E149–Q448) radioactivity peak was eluted at 0.2–0.3 mol/l NaCl from the Q-Sepharose fast-flow column and the Mono Q HR 10/10 column but at 0.1–0.2 mol/l NaCl from the Mono S HR 5/5 column.

The purity of the recombinant protein in the radioactivity peak from the Mono S column was checked by SDS/PAGE and Western blotting (see Figure 3).

RAR-β-(V7–Q448) (domains A–F)

RAR-β-(V7–Q448) was purified on Q-Sepharose, Mono Q HR 10/10 and Mono S HR 5/5 in the same way as RAR-β-(E149–Q448) except that the pH of the Mono S column was 6.2. The RAR-β-(V7–Q448) radioactivity peak was eluted at 0.25–0.50 mol/l NaCl from the Q-Sepharose fast flow column, at 0.15–0.25 ml/l NaCl from the Mono Q HR 10/10 column and at 0.15–0.20 mol/l NaCl from the Mono S HR 5/5 column (see Figure 3).

RAR-β-(P193–Q448) (domains E–F)

An attempt to purify RAR-β-(P193–Q448) by chromatography on Q-Sepharose fast flow, Mono Q HR 10/10 and Mono S HR 5/5 in the same way as RAR-β-(E149–Q448) and RAR-β-(V7–Q448) was unsuccessful, as it had a tendency to aggregate. We also tried to purify it by the same ion-exchange chromato-

graphic steps but with 6 mol/l urea in the buffers but without success.

Some degree of purification was obtained by reversed-phase column chromatography. The RAR-β-(P193–Q448) extract was applied to a ProRPC HR 5/2 column (Pharmacia) which was eluted with 0–100% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid in water. RAR-β-(P193–Q448) was eluted at about 45–60% (v/v) acetonitrile. The contents of the fractions were checked by SDS/PAGE (see Figure 3).

Size and degree of aggregation

To check the size and degree of aggregation of the recombinant proteins, all three variants of RAR-β were gel-filtered on a Superose 12 10/30 column (Pharmacia), in 20 or 50 mmol/l sodium phosphate, pH 7.4, containing 10% (v/v) glycerol, 1 mmol/l dithiothreitol (DTT) and 0.1 mmol/l (PMSF) phenylmethanesulphonyl fluoride.

RAR-β-(P193–Q448) in the form of the solubilized inclusion bodies [16] in 50 mmol/l Tris/HCl, pH 8.0, 7 mol/l guanidinium chloride, 1 mmol/l EDTA (disodium salt) and 0.1 mmol/l PMSF, was gel-filtered on a Superose 12 column, in a non-denaturing buffer system [50 mmol/l Tris/HCl, pH 8.0, 10% (v/v) glycerol, 1 mmol/l EDTA (disodium) and 0.1 mmol/l PMSF] and in a denaturing buffer system [6 mol/l guanidinium chloride, 50 mmol/l Tris/HCl, pH 8.0, 1 mmol/l EDTA (disodium) and 0.1 mmol/l PMSF]. A sample of RAR-β-(P193–Q448) in denaturing buffer was also boiled (100 °C) for 15 min before it was applied to the Superose 12 column equilibrated in the denaturing buffer system.

Determination of the amino acid sequence of the recombinant proteins

Proteins were purified as described above, subjected to SDS/PAGE and blotted on to Immobilon-P paper (Millipore). The bands containing the recombinant receptors were cut out and the amino acid sequences determined by automatized Edman degradation using an ABI protein sequencer, model 447A and an ABI analyser, model 120A.

Scatchard analysis

The receptor extracts used were a crude extract of the bacterial cells and pooled fractions containing the radioactivity peak from the Mono Q HR 10/10 column [RAR-β-(V7–Q448)], the pooled fractions containing the radioactivity peak from the Mono S column [for RAR-β-(E149–Q448)] and a preparation from the Mono Q HR 10/10 column in Tris/6 mol/l urea buffer [RAR-β-(P193–Q448)].

The receptor extracts were diluted in 0.04 mol/l Hepes, pH 7.9, containing 0.12 mol/l KCl, 10% (v/v) glycerol, 0.1% (w/v) gelatin, 1 mmol/l EDTA (disodium), 0.1 mmol/l PMSF and 4 mmol/l DTT. The chosen dilution, determined in separate experiments, was that which bound about 50% of the added [^{3}H]*all-trans*-retinoic acid.

Unlabelled retinoic acid was dissolved in dimethyl sulphoxide and serially diluted in 99.5% (v/v) ethanol (0.5 μl). [^{3}H]*all-trans*-Retinoic acid was diluted with ethanol. Then 5 μl of each dilution of unlabelled retinoic acid and 5 μl of diluted [^{3}H]*all-trans*-retinoic acid were mixed with 190 μl of diluted receptor extract and the mixture was incubated in the dark at 21 °C for 3 h or overnight. Ice-cold dextran-coated charcoal 300 μl; [2.5% (w/v) active charcoal, 0.25% (w/v) dextran T40 in 0.02 mol/l Hepes, pH 7.9, containing 0.06 mol/l KCl] was then added. The tubes were mixed, incubated at 4 °C for 15 min and then centrifuged at 4 °C at 4000 *g* for 20 min. Bound [^{3}H]*all-trans*-retinoic acid radioactivity in the supernatant was mixed with Optifluor (Packard) scintillator fluid and the ^{3}H radioactivity determined (see Figure 4).

Binding specificity of the receptor

To test the specificity of retinoids for the receptor, the following binding experiments were performed: 2–3 nmol/l [^{3}H]*all-trans*-retinoic acid (5 μl), various concentrations of unlabelled retinoids (5 μl) and 190 μl of receptor extract in 0.04 mol/l Hepes, pH 7.9, containing 0.12 mol/l KCl, 10% (v/v) glycerol, 0.1% (w/v) gelatin, 1 mmol/l EDTA (disodium), 0.1 mmol/l PMSF and 4 mmol/l DTT. The extracts were the same as those used in the Scatchard analysis except for the experiments on RAR-β-(V7–Q448), which were performed with the pooled fractions containing the radioactivity peak from the Mono S HR 5/5. Some experiments were also carried out with crude bacterial extracts. Incubation was in glass tubes in the dark at 21 °C for 3 h or at 4 °C for 20 h. Ice-cold dextran-coated charcoal (see above) was then added and the tubes were kept for 15 min at 4 °C. The tubes were centrifuged at 4 °C at 4000 *g* for 20 min and the supernatants were assayed for radioactivity (see above) (see Figure 5). In some cases the volume was scaled up to allow addition of larger volumes of unlabelled retinoid (see Figure 7) or the quantification by HPLC of the retinoids remaining in the supernatant after treatment with dextran-coated charcoal (see Table 2).

RESULTS

The concentration of RAR-β-(V7–Q448), RAR-β-(E149–Q448) and RAR-β-(P193–Q448) of bacterial cell suspension was approx. 5–10, 25–30 and about 40% of total protein respectively, as judged visually from the Coomassie staining of SDS/polyacrylamide gels (Figure 2). In our hands the BL21(DE3)pLysS cells gave higher yields of RAR-β-(P193–Q448) than the BL21(DE3) cells, for RAR-β-(V7–Q448) there was no difference and for RAR-β-(E149–Q448) the BL21(DE3) gave the best yields. Rifampicin improved the yields of both RAR-β-(E149–Q448) and RAR-β-(P193–Q448) but not of RAR-β-(V7–Q448). A period of 3 h of induction at 37 °C with IPTG was found to be optimal.

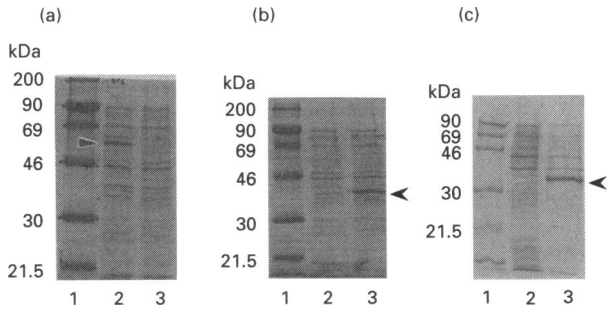

Figure 2 Coomassie Blue staining of gels after SDS/PAGE of the recombinant proteins expressed in *E. coli*

(**a**) Cell suspension before (lane 3) and after (lane 2) induction of RAR-β-(V7–Q448) with IPTG. Lane 1, molecular-mass standard. (**b**) Cell suspension before (lane 2) and after (lane 3) induction of RAR-β-E149–Q448). Lane 1, molecular-mass standard. (**c**) Cell suspension before (lane 2) and after (lane 3) induction of RAR-β-(P193–Q448). Lane 1, Molecular-mass standard.

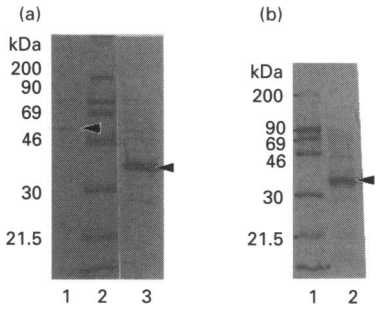

Figure 3 Purification of RAR-β-(V7–Q448), RAR-β-(E149–Q448) and RAR-β-(P193–Q448)

Purification of RAR-β-(V7–Q448) and RAR-β-(E149–Q448) was by chromatography on Q-Sepharose, Mono Q and Mono S and that of RAR-β-(P193–Q448) was by chromatography on ProRPC. After SDS/PAGE, the gels were stained with Coomassie Blue. (**a**) Lane 1, RAR-β-(V7–Q448) (pooled fractions from the Mono S column); lane 2, molecular-mass standards; lane 3, RAR-β-(E149–Q448) (pooled fractions from the Mono S column). (**b**) Lane 1, molecular-mass standard; lane 2, RAR-β-(P193–Q448) (pooled fractions from the ProRPC column).

The recombinant receptors were produced mainly as inclusion bodies. To purify the inclusion bodies and to extract and renature the recombinant proteins, we followed the procedure described by Lin et al. [16]. The final supernatants contained the renatured RAR-β derivatives. SDS/PAGE with Western blotting (not shown) showed that RAR-β-(V7–Q448), RAR-β-(E149–Q448) and RAR-β-(P193–Q448) constituted roughly 5, 40 and 50% respectively of the total protein in the crude bacterial extract as judged visually from Coomassie Blue staining of the gels, or about 3 mg/l for RAR-β-(V7–Q448) and about 100 mg/l for RAR-β-(E149–Q448) (Figure 3).

Chromatography of the RAR-β-(E149–Q448) and RAR-β-(V7–Q448) on Q-Sepharose, Mono Q and Mono S after addition of [^{3}H]*all-trans*-retinoic acid gave distinct radioactivity peaks, the elution position of which corresponded to the position of the recombinant protein. RAR-β-(E149–Q448) was eluted from Mono S at an NaCl concentration of 0.1–0.2 mol/l and RAR-β-(V7–Q448) at an NaCl concentration of 0.15–0.20 mol/l. The receptors were purified to about 50–70% of total protein with these three chromatographic steps (Figure 3). The concentrations in these extracts were about 1 mg/l RAR-β-(V7–Q448) and 10 mg/l RAR-β-(E149–Q448). We tried to purify RAR-β-(P193–Q448) in the same way as the other recombinant receptors and also under denaturing conditions with the same chromatographic steps, all with limited success. Some degree of purification was obtained with a ProRPC column. The expected size of the recombinant receptors was confirmed by SDS/PAGE (Figures 2 and 3).

To find out whether the different recombinant receptors were in monomeric or oligomeric/multimeric form, extracts of the respective proteins were gel filtered on Superose 12 at pH 7–8 under denaturing and non-denaturing conditions (not shown). Under non-denaturing conditions RAR-β-(E149–Q448) (molecular mass about 36 kDa) was eluted with a $K_{av.}$ of 0.38 which roughly corresponds to its calculated monomeric molecular mass. RAR-β-(V7–448) was eluted with a $K_{av.}$ similar to ovalbumin (molecular mass about 43 kDa), which indicates a monomeric state. RAR-β-(P193–Q448), in contrast, was eluted in the void volume as what seems to be a multimer, corresponding to a molecular mass of more than 100 kDa (expected molecular mass about 33 kDa). When RAR-β-(P193–Q448) was gel-filtered under denaturing conditions it was eluted partly as what may be

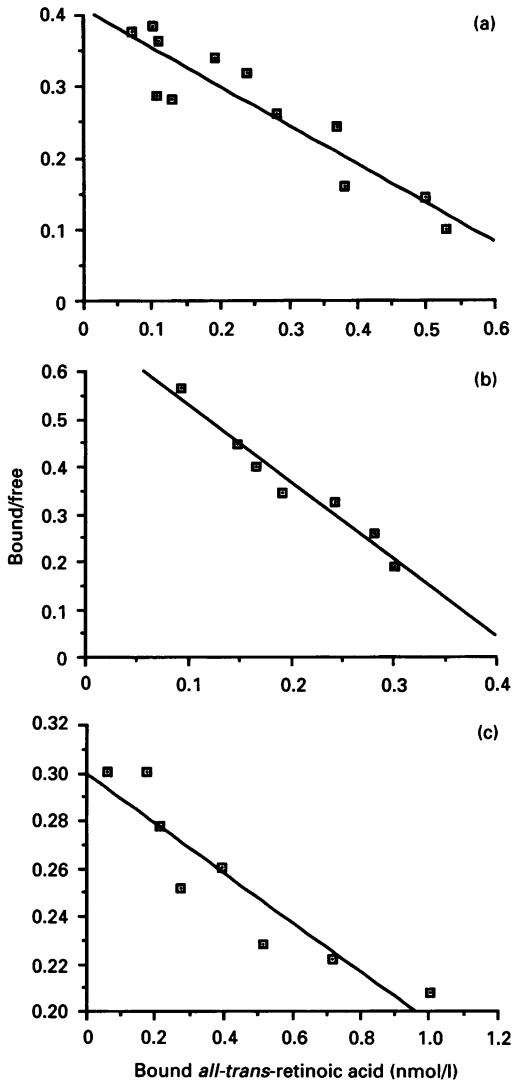

Figure 4 Binding affinity of the three variants of recombinant RAR-β for *all-trans*-retinoic acid

(**a**) Scatchard plot of RAR-β-(V7–Q448). The regression of the line was $y = 0.47–0.69x$; $r^2 = 0.93$. K_d was 1.4 nmol/l and the receptor concentration receptor 0.7 nmol/l. (**b**) Scatchard plot of RAR-β-(E149–Q448). The regression of the line was $y = 0.69–1.62x$; $r^2 = 0.96$. K_d was 0.6 nmol/l and receptor concentration 0.4 nmol/l. (**c**) Scatchard plot of RAR-β-(P193–Q448). The regression of the line was $y = 0.30–0.10x$; $r^2 = 0.85$. K_d was 9.6 nmol/l and the receptor concentration 2.9 nmol/l.

a monomer ($K_{av.} = 0.27$) but also as a di- or multi-mer ($K_{av.} = 0.18$). If the solution of RAR-β-(P193–Q448) was denatured and boiled (100 °C) for 15 min before it was applied to the Superose 12 column (in denaturing buffer), it was eluted almost exclusively at a $K_{av.}$ of 0.27 which would be expected for a monomer.

Determination of the sequence of the N-terminus of RAR-β-(E149–Q448) confirmed the sequence of the first eight amino acids, and for the N-terminus of RAR-β-(P193–Q448) the sequence of the first five amino acids was confirmed.

From the Scatchard plots (Figure 4) the K_d for the RAR-β-(E149–Q448)–*all-trans*-retinoic acid complex was calculated to be 0.6 nmol/l, that for the RAR-β-(V7–Q448)–*all-trans*-retinoic acid complex to be 1.4 nmol/l and that for the RAR-β-(P193–Q448)–*all-trans*-retinoic acid complex to be 9.6 nmol/l.

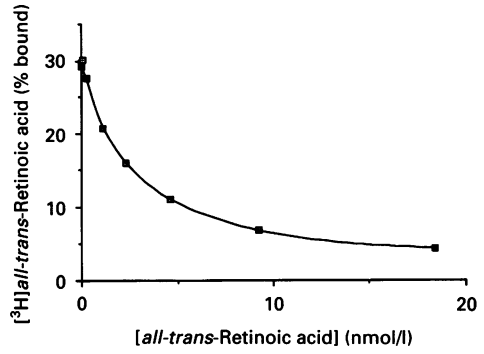

Figure 5 Standard curve for *all-trans*-retinoic acid with RAR-β-(E149–Q448) as binder

Incubation conditions and volumes were as described for the Scatchard plots (190 μl of receptor extract, 5 μl of [³H]*all-trans*-retinoic acid and 5 μl of unlabelled *all-trans*-retinoic acid). The concentration of the *X*-axis is the concentration of the unlabelled *all-trans*-retinoic acid in the 5 μl.

The data shown in Figure 4(b) for RAR-β-(E149–Q448) are also plotted as a standard curve (Figure 5) to show that the possible measuring range of an assay based on recombinant RAR-β-(E149–Q448) encompasses the concentration range of *all-trans*-retinoic acid in the plasma [1,2].

The retinoid specificity of the recombinant receptors was studied in a relative receptor-binding assay, where [³H]*all-trans*-retinoic acid was allowed to compete with different unlabelled retinoids (Figure 6, Table 1). It was found that RAR-β-(V7–Q448) and RAR-β-(E149–Q448) both bound 9-*cis*-retinoic acid as well as *all-trans*-retinoic acid. Except for 13-*cis*-retinoic acid, 4-oxo-*all-trans*-retinoic acid and retinol, the other retinoids were weakly bound by RAR-β-(V7–Q448) and RAR-β-(E149–Q448).

13-*cis*-Retinoic acid is present in serum at about the same concentration as *all-trans*-retinoic acid, and *all-trans*-retinol at a concentration 500 times that of *all-trans*-retinoic acid. The concentration of 4-oxo-*all-trans*-retinoic acid is low [17]. Therefore we decided to characterize the binding of 13-*cis*-retinoic acid and retinol to RAR-β-(E149–Q448) in more detail. This was done by incubating crude extracts of RAR-β-(E149–Q448) (receptor concentration was determined from a Scatchard plot) with [³H]*all-trans*-retinoic acid and more than 1000 × unlabelled *all-trans*-retinoic acid, 13-*cis*-retinoic acid or retinol. The volume given in the Materials and methods section was scaled up 2.5 times to allow HPLC quantification of the retinoids remaining after dextran/charcoal treatment (Table 2). The retinoid present in the supernatant after the incubation of recombinant receptor with a large excess of *all-trans*-retinoic acid was *all-trans*-retinoic acid. Most of the [³H]*all-trans*-retinoic acid was displaced by the unlabelled *all-trans*-retinoic acid (Table 2). After incubation with 13-*cis*-retinoic acid in large excess, more *all-trans*-retinoic acid than 13-*cis*-retinoic acid was found in the supernatant. After incubation with more than 1000-fold excess of retinol, only retinol could be demonstrated in the supernatant (Table 2).

We also studied the binding of [³H]*all-trans*-retinol by serial dilutions of the crude receptor extract (not shown) and found binding virtually independent of receptor dilution.

We added increasing concentrations of unlabelled *all-trans*-retinol to a standard curve consisting of ³H-labelled and unlabelled *all-trans*-retinoic acid and found that increasing

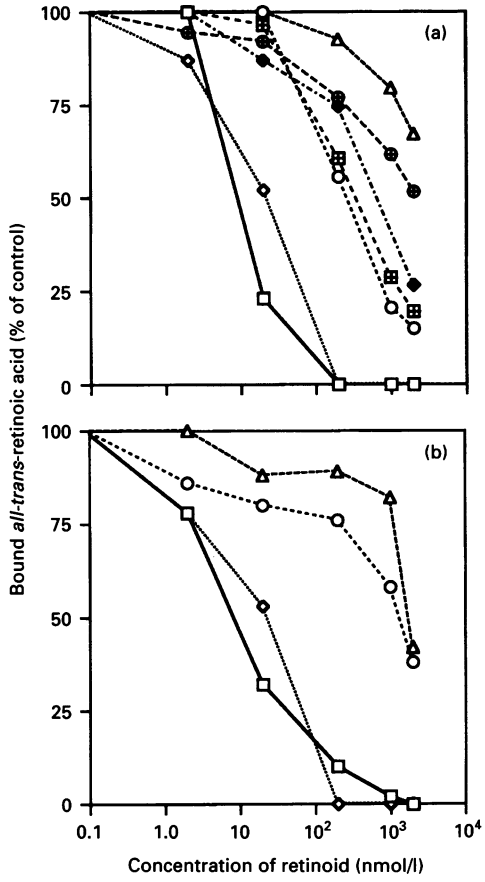

Figure 6 Binding specificity of recombinant RAR-β-(E149–Q448) and RAR-β-(V7–Q448)

(**a**) RAR-β-(E149–Q448) specificity for *all-trans*-retinoic acid (□), 9-*cis*-retinoic acid (◇), 13-*cis*-retinoic acid (○), 4-oxo-13-*cis*-retinoic acid, (△), 4-oxo-*all-trans*-retinoic acid (⊞), retinol (◆) and acitretin (⊕). Increasing concentrations of different unlabelled retinoids were added to receptor extract, labelled with ³H-*all-trans*-retinoic acid. Conditions were as described in the text. (**b**) RAR-β-(V7–Q448) specificity for *all-trans*-retinoic acid (□), 9-*cis*-retinoic acid (◇), 13-*cis*-retinoic acid (○) and 4-oxo-13-*cis*-retinoic acid (△). The conditions were the same as in (**a**).

concentrations of unlabelled *all-trans*-retinoic acid (Figures 7a and 7b) diminished [³H]*all-trans*-retinoic acid binding.

Finally we tested the hypothesis that impurities (unidentified peaks which were more abundant in older batches of *all-trans*-retinol) in the retinol preparation, which could be seen with the sensitive HPLC system used [4], were responsible for the displacement of *all-trans*-retinoic acid from the receptor. We incubated crude extracts of RAR-β-(E149–Q448) with [³H]*all-trans*-retinoic acid and approx. 100–400-fold excess of *all-trans*-retinol preparations with impurities varying from 0.5 to 5% over *all-trans*-retinoic acid (not shown). The results clearly show that the *all-trans*-retinol, and not the impurities, was responsible for the displacement.

DISCUSSION

We tried several expression systems and eventually chose the expression vector pET-3d/BL21(DE3), as it has been shown to express this class of receptors in good yield [16,18].

Three variants of RAR-β were expressed: RAR-β-(V7–Q448) (Val^7–Gln^{448}) corresponding to domains A–F in Figure 1, RAR-

Table 1 Retinoid specificity of the RAR-β-(E149–Q448) receptor

Purified RAR-β-(E149–Q448) was incubated with 2 nmol/l [^{3}H]*all-trans*-retinoic acid and 1000 nmol/l unlabelled retinoid as described in the text. Binding of [^{3}H]*all-trans*-retinoic acid with no added retinoid was taken as 100%

Addition	Binding of [^{3}H]*all-trans*-retinoic acid (%)
None	100
all-trans-Retinoic acid	0
9-*cis*-Retinoic acid	0
13-*cis*-Retinoic acid	20
4-oxo-13-*cis*-Retinoic acid	79
4-oxo-*all-trans*-Retinoic acid	28
Retinol	42
Acitretin	61

Table 2 Displacement of [^{3}H]*all-trans*-retinoic acid by *all-trans*-retinoic acid, 13-*cis*-retinoic acid and *all-trans*-retinol from binding to RAR-β-(E149–Q448) and quantification of the retinoids remaining after adsorption with dextran-coated charcoal

RAR-β-(E149–Q448) (3.36 μmol/l), [^{3}H]*all-trans*-retinoic acid (2–3 μmol/l) and the respective retinoid (3.36 μmol/l) were incubated in a total volume of 400 μl. Buffers and other additions were as described in the Materials and methods section but scaled up by a factor of 2.5. Incubation was at 4 °C for 20 h. Dextran-coated charcoal (600 μl) was then added. A fraction of the supernatant was removed for measurement of ^{3}H radioactivity and the rest was analysed by HPLC [4]. Data from measurements in a blank incubation (with buffer instead of receptor) were subtracted. Results from incubations with receptor and tracer only (i.e. no added retinoid) were set to 100%. ND, not determined.

Additions	[^{3}H]*all-trans*-Retinoic acid (%)	Concentration in supernatant (nmol/l)		
		all-trans-Retinoic acid	13-*cis*-Retinoic acid	*all-trans*-Retinol
RAR-β-(E149–Q448) + *all-trans*-retinoic acid (3.36 μmol/l)	3.2	8.75	0.25	–
RAR-β-(E149–Q448) + 13-*cis*-retinoic acid (3.36-μmol/l)	28.7	4.23	0.87	–
RAR-β-(E149–Q448) + *all-trans*-retinol (3.36 μmol/l)	ND	–	–	8.0

β-(E149–Q448), the ligand-binding and hinge domains D–F (Glu^{149}–Gln^{448}) and RAR-β-(P193–Q448), the ligand-binding domain E–F (Pro^{193}–Gln^{449}). Most of the receptor protein was expressed in inclusion bodies.

RAR-β-(P193–Q448) forms aggregates and behaves as a very hydrophobic protein in most systems and does not bind *all-trans*-retinoic acid very efficiently (K_d = 9.6 nmol/l). The difference between RAR-β-(E149–Q448) and RAR-β-(P193–Q448) is that the former contains 44 extra amino acids, from the D region (Figure 1), the function of which are unknown at present. However, the D region seems to be important for the ligand binding by either directly participating in the binding or indirectly blocking aggregation. A hydrophilicity/hydrophobicity plot [19,20] of RAR-β (not shown) shows a high degree of hydrophobicity in the ligand-binding (E + F) domain, whereas the D domain has a more hydrophilic character, which may explain the tendency of RAR-β-(P193–Q448) (which does not contain the D region) to form aggregates. There is also a dimerization site in domain E [21,22] which might increase the tendency for aggregation, which could be masked or blocked by the D domain in RAR-β-(E149–Q448).

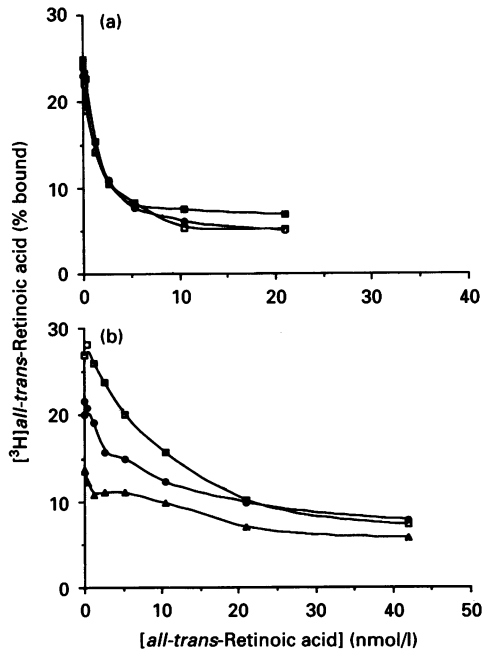

Figure 7 Effect of retinol on binding of [^{3}H]*all-trans*-retinoic acid by RAR-β-(E149–Q448)

To aliquots of diluted crude extract of RAR-β-(E149–Q448) (0.42 nmol/l, final concentration) and tracer {2–3 nmol/l [^{3}H]*all-trans*-retinoic acid (□)} was added increasing concentrations of unlabelled *all-trans*-retinoic acid and 4.2 (○), 42 (■), 210 (●) or 420 (▲) nmol/l retinol (final concentrations). The total volume was 450 μl and the retinoids were added dissolved in ethanol (final concentration of ethanol 10% v/v). (**a**) Effect of 4.2 and 42 nmol/l retinol; (**b**) effect of 210 and 420 nmol/l retinol.

Crettaz et al. [8] showed that a 28 kDa fragment of RAR-β, consisting of the ligand-binding domain (domains E–F?), had a similar K_d for *all-trans*-retinoic acid to that of whole RAR-β. In their experiments they used a crude bacterial extract of receptor, which was not purified further. This may indicate that the problem with aggregation that we have encountered with the shorter ligand-binding domain [RAR-β-(P193–Q448), E–F] might be an artifact of the purification process.

RAR-β-(E149–Q448) (the D–F domains) bound *all-trans*-retinoic acid with about the same avidity (within experimental error) as RAR-β-(V7–Q448) (the A–F domains), indicating that the A–C domains do not influence ligand binding appreciably. The affinity of RAR-β-(E149–Q448) for retinoids was such that it could be used as a binder for *all-trans*-retinoic acid (Figure 5). RAR-β-(V7–Q448) and RAR-β-(E149–Q448) were both obtained as monomeric receptors in solution and showed good binding of *all-trans*-retinoic acid (K_d 1.4 and 0.6 nmol/l). These K_d values are of the same magnitude as found previously [8,23]. RAR-β-(E149–Q448) and RAR-β-(V7–Q448) were also good binders of 9-*cis*-retinoic acid [24] (Figure 6). These receptors also seemed to bind 13-*cis*-retinoic acid. This is, however, probably an artifact as we have found that 13-*cis*-retinoic acid is never completely pure after being handled; there is always a small amount of *all-trans*-retinoic acid in our preparations. The experiments in Table 2 demonstrate the same point. This explains most of the 'binding' of 13-*cis*-retinoic acid by the receptor shown in Figure 6. Thus 13-*cis*-retinoic acid is probably not bound by RAR-β, in accord with the findings of Levin et al. [25].

In accord with an earlier report [26], we find that 4-oxo-*all-trans*-retinoic acid binds weakly to RAR-β-(E149–Q448), and 4-oxo-13-*cis*-retinoic acid binds much less well.

We cannot explain the apparent weak binding of *all-trans*-retinol to RAR-β-(E149–Q448). Similar results have been reported by others [8,23] but not explored further. Besides weak binding of *all-trans*-retinol itself to RAR-β-(E149–Q448), another possible explanation is that commercial preparations of *all-trans*-retinol contain trace amounts of some other component that has the ability to compete with *all-trans*-retinoic acid for binding to RAR-β-(E149–Q448). We tested the latter hypothesis and found that *all-trans*-retinol displaces *all-trans*-retinoic acid from binding to RAR-β-(E149–Q448) (results not shown). The concentration of *all-trans*-retinol in serum is several hundred times higher than the concentration of *all-trans*-retinoic acid. The same may be the case in the tissues. It is not known whether competition between *all-trans*-retinoic acid and *all-trans*-retinol for binding to retinoid receptors occurs physiologically.

Serum contains low concentrations of the 4-oxo- metabolites and very low concentrations of 9-*cis*-retinoic acid (M. Berggren Söderlund, G. Johannesson and G. Fex, unpublished work). Some 13-*cis*-retinoic acid is, however, present normally, at a concentration approximately equimolar with *all-trans*-retinoic acid. Serum retinol concentration is about 500 times that of *all-trans*-retinoic acid; some of the retinol has to be removed before assay of *all-trans*-retinoic acid with RAR-β-(E149–Q448) as binder.

The observed avidity and specificity of RAR-β-(E149–Q448) for retinoids makes this recombinant protein an interesting candidate as a binder in a ligand-binding assay for *all-trans*-retinoic acid.

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