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Ligand binding properties of human cellular retinoic acid binding protein II expressed in *E. coli* as a glutathione-*S*-transferase fusion protein

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To test the hypothesis that 9-*cis*-retinoic acid is a ligand for cellular retinoic acid binding protein II (CRABP II), human CRABP II was expressed as a glutathione-*S*-transferase fusion protein (GST-CRABP II) and a single affinity purification step used to extract it from bacterial lysates. GST-CRABP II bound *all trans*-retinoic acid with high affinity (K_d 14.2 ± 6.5 nM), but 9-*cis*-retinoic acid bound poorly. These studies suggest that 9-*cis*-retinoic acid is not a ligand for CRABP II. Their ease of purification makes GST-CRABP fusion proteins useful tools for ligand binding studies with different retinoids.

Retinoic acid; CRABP II; Ligand binding; Fusion protein; Glutathione-*S*-transferase

1. INTRODUCTION

Cellular retinoic acid binding protein (CRABP) is a low molecular weight ($M_r \approx 16{,}000$) cytoplasmic protein which binds *all trans*-retinoic acid with high affinity and is expressed in a variety of tissues [1]. Recently, two forms of CRABP have been identified [2–4]; while closely related in sequence they have distinct biophysical properties and are reported to differ in their affinity for *all trans*-retinoic acid, with CRABP I having a higher affinity than CRABP II [5,6]. CRABP may be of critical importance in development by regulating the availability of *all trans*-retinoic acid to the nuclear retinoic acid receptors (RARs).

RARs are ligand-dependent transcriptional regulators that bind *all trans*-retinoic acid with high affinity and represent a subset of the steroid/thyroid hormone receptor superfamily [7]. A distinct yet closely related group of nuclear receptors, termed retinoid X receptors or RXRs [8], has been shown to bind 9-*cis*-retinoic acid [9,10]. RXRs have the potential to regulate diverse hormone-response pathways by acting as heterodimers with the nuclear receptors for thyroid hormone, vitamin D and *all trans*-retinoic acid [11]. Thus, 9-*cis*-retinoic acid may be an important intracellular regulatory molecule, but very little is yet known of its pathways of synthesis, metabolism and transport within the cell. The fact that CRABP II apparently has a 15-fold lower affinity for *all trans*-retinoic acid than CRABP I [5] raises the possibility that CRABP II could be a specific 9-*cis*-retinoic acid binding protein. To test this hypothesis, we have cloned the coding sequence for human CRABP II into an expression vector and expressed it in *E. coli* as a glutathione-*S*-transferase (GST) fusion protein. We report the ligand binding properties of GST-CRABP II and show that, by comparison with *all trans*-retinoic acid, the 9-*cis* isomer displays a low affinity for CRABP II.

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2. MATERIALS AND METHODS

2.1. *Retinoic acid*

Stock solutions of *all trans*-retinoic acid (Sigma) and 9-*cis*-retinoic acid (a gift from Dr. M. Klaus, Hoffman-La Roche, Basel) were made up in ethanol assuming an extinction coefficient of 36,500 $M^{-1} \cdot cm^{-1}$ at 343 nm. The radiochemical purity of *all trans*-[11,12-^{3}H]retinoic acid (49.3 Ci/mmol, Du Pont-NEN, Stevenage, UK) was verified by isocratic elution (2 ml min^{-1}) from a 25 cm × 4.6 mm Zorbax-NH_2 (Du Pont) column in 90:10 acetonitrile:dichloromethane, 10 mM acetic acid. All manipulations of retinoic acid were done under darkroom- or dim-light conditions; stock solutions were stored at −20°C in the dark and used within 2–3 days.

2.2. *Cloning and sequence analysis of human CRABP II*

The CRABP II coding sequence was amplified from 1 μg total RNA from human retinoic acid-treated dermal fibroblasts [12]. cDNA was synthesised by adding RNA to 200 μM of each deoxynucleotide triphosphate, 0.32 μg oligo d(T) primer, 5 mM $MgCl_2$, 2.5 U RNasin, and 15 U AMV reverse transcriptase (Promega, Southampton, UK) in a total volume of 20 μl, buffered with material supplied with the enzyme. Incubation for 15 min at 42°C was followed by denaturation at 95°C, 10 min; after chilling on ice, 10 μl of the cDNA reaction was added to 100 pmol primer 1 (5'ATTCTAGATGCCCAACTTCTC-TGGCAACTG 3', *Xba*I site underlined, initiation ATG in bold), 100 pmol primer 2 (5'CTGGATCCTCACTCTCGGACGTAGACCCT-G3', *Bam*HI site underlined, stop codon in bold), 2 mM $MgCl_2$, and 2.5 U *Taq* polymerase (Perkin-Elmer-Cetus) in a total volume of 100

μl containing buffer supplied with the enzyme. Amplification was for 30 cycles of denaturation at 95°C (30 s), annealing at 55°C (30 s) and extension at 72°C (42 s). Primer sequences were based on the human CRABP II sequence reported by Eller et al. [3]. PCR products (ca. 420 bp) were digested with *Xba*I and *Bam*HI and cloned into Bluescript. The CRABP II cDNA sequence was verified by sequencing the insert in both directions with an Applied Biosystems DNA sequencer. CRABP II was re-amplified from the plasmid with new primers to incorporate *Bam*HI and *Eco*RI sites (primer 3: 5'end, 5'ACTGGATCCATGCCCAACTTCTCTGGCAAC 3', *Bam*HI site; primer 4: 3' end, 5'GATGAATTCCTCACTCTCGGACGTAGAC3', *Eco*RI site) and subcloned into the pGEX-2T vector at the *Bam*HI/*Eco*RI sites in-frame with the GST coding sequence and immediately downstream of the thrombin cleavage site; cleavage of the GST-CRABP II fusion protein with thrombin will thus yield a CRABP II peptide with a Gly-Ser amino-terminal extension. Recombinant plasmid (pG-CRABP2) was transformed into *E. coli* DH5α and the expression of GST-CRABP II fusion protein verified by SDS-PAGE [13]: overnight cultures in LB medium [14] containing 50 $\mu g \cdot ml^{-1}$ ampicillin were diluted 1:10 with fresh medium, isopropyl-β-D-thiogalactopyranoside (IPTG, Sigam) was added to 0.1 mM 1 h later and the cells harvested after a further 4 h at 37°C [15].

2.3. *Extraction and affinity gel purification of GST-CRABP II fusion protein*

GST-CRABP II fusion protein was extracted from 1 l cultures as described by Quinn et al. [15]. Briefly, after induction of fusion protein with IPTG (as above), cells were harvested, washed with 25% w/v sucrose, 10 mM EDTA, 0.1 mM PMSF, 50 mM Tris-HCl, pH 8.0 (buffer A), and resuspended in 40 ml buffer A containing 2 $mg \cdot ml^{-1}$ lysozyme. After 1 h on ice, cells were resuspended in 20 ml 14 mM Na_2HPO_4/8 mM KH_2PO_4, pH 7.0 (buffer B), and lysed by freeze-thawing twice. Bacterial debris was removed by centrifugation and the lysate mixed with 5 ml GSH-Sepharose 6B beads (50% v/v) [16], for 1–2 h at 4°C. Beads carrying adsorbed fusion protein were spun down (1 000 × *g*, 1 min at 4°C) and washed 5 times with buffer B and once in 50 mM Tris-HCl, pH 8.0. Fusion protein was eluted by resuspending the beads for 1 h at room temperature in an equal volume of 10 mM GSH, 50 mM Tris-HCl, pH 8.0. The supernatant was concentrated by ultrafiltration through Centricon 10 U (Amicon, Stonehouse, UK) and the fusion protein stored at −20°C either frozen or in 50% glycerol. As a control for experiments with the GST-CRABP II fusion protein, GST protein was extracted as above from IPTG-induced cultures of *E. coli* DH5α carrying the pGEX-2T plasmid.

Cleavage of the fusion protein was by incubation, either in solution or bound to the GSH affinity gel beads, for 1 h at room temperature with an excess (10 $U \cdot ml^{-1}$ beads or 0.17 $U \cdot \mu g^{-1}$ fusion protein) of thrombin (Calbiochem, 3554 $U \cdot mg^{-1}$) in 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1% 2-mercaptoethanol, 50 mM Tris-HCl, pH 8.0 (buffer C).

2.4. *Protein estimation and SDS-PAGE*

Protein concentrations were estimated from BSA standards (Sigma) using the Bradford method [17]. 15% acrylamide gels (0.75 mm thick) were run to a length of 6 cm using the Laemmli buffer system [13]. Gels were fixed and stained with 0.05% Coomassie blue in 20% methanol, 10% acetic acid.

2.5. *High performance size-exclusion chromatography (HPSEC)*

HPSEC of *all trans*-[11,12-^{3}H]retinoic acid bound to fusion protein was by elution (1 $ml \cdot min^{-1}$) from a Phenomenenex (Macclesfield, UK) Biosep SEC S3000 column (300 × 4.6 mm) in 50 mM sodium phosphate buffer, pH 6.8, at room temperature. Eluate fractions were collected for scintillation counting.

2.6. *Fluorimetric titration*

The binding of retinoic acid to GST-CRABP II fusion protein, cleaved CRABP II and GST protein was monitored by fluorimetric titration [18,19] at room temperature with a Kontron SFM 25 fluorimeter set to excitation and emission wavelengths of 350 and 475 nm, respectively. Retinoic acid was added in ethanol (1–2 μl per addition) to 2 ml 0.2–1 μM GST-CRABP II fusion protein, 50 mM Tris-HCl, pH 7.5, in borosilicate glass tubes, and mixed by inversion. After each addition, the ligand was allowed to bind for 5 min before inserting the tube into the fluorimeter. Once the fluorimeter reading had stabilised (ca. 6 s), the mean fluorescence over two consecutive 8-s periods was recorded using the integrator. Fluorescence readings were stable over this period but declined with longer exposure in the fluorimeter; tubes were thus exposed to UV within the fluorimeter for the shortest possible time. The final ethanol concentration was less than 1%. To compare the binding of *all trans*- and 9-*cis*-retinoic acid, a different approach was used to minimise the possibility of UV-induced *cis*→*trans* isomerization of 9-*cis*-retinoic acid: retinoic acid at an increasing concentration was added to each of a series of tubes containing 1.2 ml GST-CRABP II fusion protein in 50 mM Tris-HCl, pH 7.5, and the fluorescence recorded once for each tube after allowing the ligand to bind for > 5 min. Binding affinity for the ligand was estimated by plotting αP_0 against $(\alpha/1-\alpha)R_0$ where P_0 is binding protein concentration (M), R_0 is retinoid concentration (M) and α is $F_{max}-F/F_{max}-F_0$ where F_{max} is the maximum fluorescence intensity (at saturation), F_0 is the initial fluorescence intensity in the absence of retinoic acid and F is the fluorescence intensity at a particular R_0 [18,19]. Excitation and emission spectra were recorded in 50 mM Tris-HCl, pH 7.5, using a quartz cell in a Perkin-Elmer LC3 fluorimeter.

3. RESULTS

3.1. *Expression of human CRABP II as a GST fusion protein*

The 422 bp human CRABP II cDNA amplified from fibroblast RNA extended from the ATG initiation codon to the TGA stop and the nucleotide sequence was identical to that previously reported for human CRABP II [3,4] but with two exceptions: a G replacing an A at

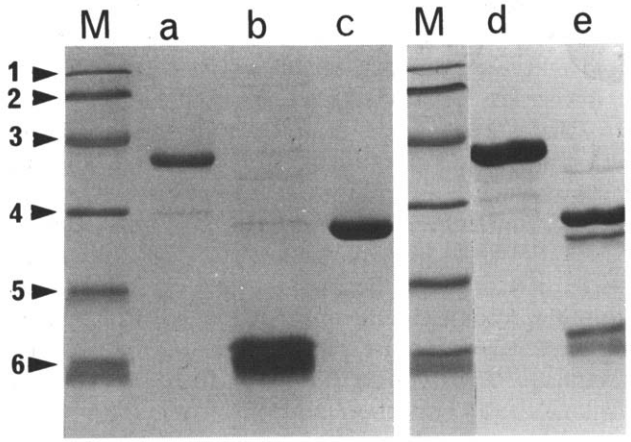

Fig. 1. SDS-PAGE (15% acrylamide) of affinity-purified GST-CRABP II. Track: (a) GST-CRABP II fusion protein from pG-CRABP2 transformants after elution from affinity gel; (b) thrombin cleavage products from GST-CRABP II bound to affinity gel; (c) affinity-purified GST expressed in *E. coli* from pGEX-2T; (d) GST-CRABP II after incubation for 1 h at room temperature (control for track e); (e) GST-CRABP II digested with an excess of thrombin (5 U/30 μg protein) for 1 h at room temperature; (M) marker proteins: 1, phosphorylase b (M_r 94 400); 2, BSA (M_r 66 200); 3, ovalbuman (M_r 45 000); 4, bovine carbonic anhydrase (M_r 31 000); 5, soybean trypsin inhibitor (M_r 21 500); 6, lysozyme (M_r 14 400).

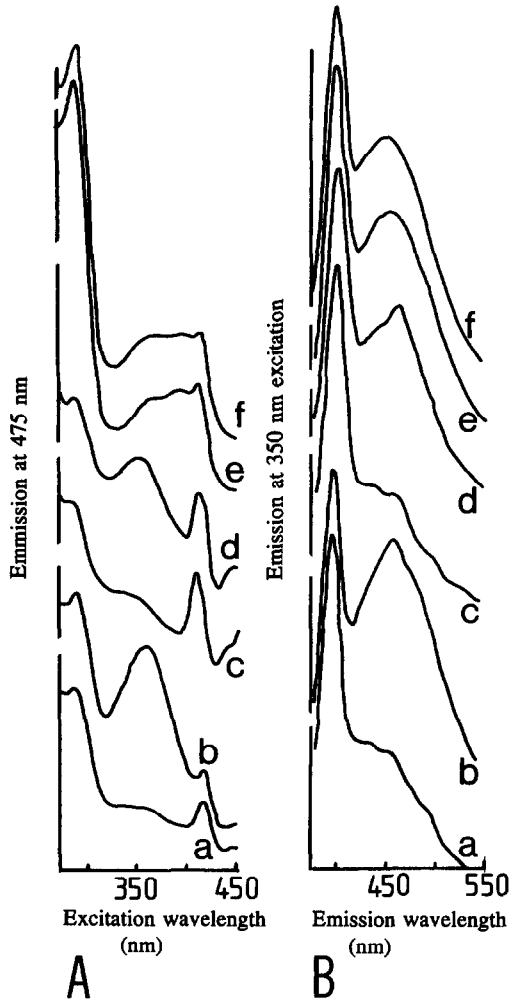

Fig. 2. Fluorescence excitation (A) and emission (B) spectra for GST-CRABP II fusion protein (a,b, nominally 1 μM), thrombin-cleaved CRABP II (c,d, 0.2 μM), and GST (e,f, 2 μM), in the presence (b,d and f) and absence (a,c and e) of 0.3 μM *all trans*-retinoic acid.

the second position of codon 71, resulting in a Glu→Gly amino acid substitution, and a C instead of T at the third base of codon 129 with no change in amino acid sequence. Human CRABP II consists of 138 amino-acid residues and has a predicted $M_r \approx 15\,700$ [3,4].

Insertion of the CRABP II coding sequence into pGEX-2T, in-frame with the GST gene, resulted in the production of a fusion protein of apparent M_r 43 400 in IPTG-induced cells carrying the pG-CRABP2 plasmid. GST expressed in *E. coli* from pGEX-2T has an apparent M_r of 27,500 [20] (Fig. 1) and the size of fusion protein in pG-CRABP2 transformants was therefore consistent with expression of a full-length GST-CRABP II fusion protein. A single step, affinity gel purification of lysates from cultures expressing pGEX-2T or pG-CRABP,2 yielded the M_r 27 500 or M_r 43,400 GST-CRABP II proteins, respectively, as the major products with only minor contamination with other proteins (Fig. 1). The yield of GST-CRABP II was 2.5–3.5 mg · l^{-1} culture. Cleavage of the GST-CRABP II protein bound to the affinity gel produced three closely spaced protein bands in the eluate, with the larger band having an apparent M_r of 15 200, similar to that expected for human CRABP II (Fig. 1). The smaller cleavage products (apparent M_r 14 400 and 13 800) may have resulted from partial cleavage within CRABP II at arginine residues 112 and 133 towards the carboxy-terminus of the protein.

3.2. *Binding of* all trans-*retinoic acid to GST-CRABP II fusion protein*

The binding of *all trans*-retinoic acid to GST-CRABP II fusion protein was assessed by fluorimetry. Excitation and emission spectra showed that adding *all trans*-retinoic acid to the fusion protein produced an increased emission at 475 nm with excitation wavelengths of 350–360 nm, consistent with the specific binding of retinoic acid (Fig. 2). Similar changes were observed after adding *all trans*-retinoic acid to CRABP II eluted from the affinity gel after cleavage with thrombin (Fig. 2). Conversely, no changes in excitation or emission spectra were produced by adding retinoic acid to GST protein alone (Fig. 2). HPSEC analysis of the fusion protein after incubation with *all trans* [11,12-^{3}H]retinoic

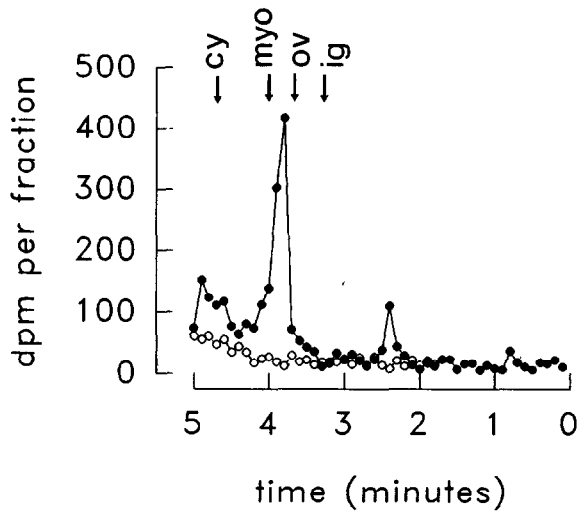

Fig. 3. HPSEC of 0.6 μg GST-CRABP II after incubation with 8 nM *all trans*-[11,12-^{3}H]retinoic acid in the presense (●) and absence (○) of a 200-fold excess of unlabelled retinoic acid. Before injection onto the column, free [^{3}H]retinoic acid was removed by treating the fusion protein (6 μg in 200 μl 8 nM [^{3}H]retinoic acid) with an equal volume of dextran-coated charcoal [24] and 40 μl of the supernatant injected. Peak (a), eluted in the excluded volume at 2.4 min, presumably represents high molecular weight aggregates; the major peak (b) at 3.8 min was eluted at the position expected for a M_r 43 400 protein, Peak(s) at 4.5–4.9 min represent free retinoic acid released from GST-CRABP II during HPSEC. Marker proteins are IgG (ig, M_r 150 000), ovalbumin (ov, M_r 45 000), myoglobin (myo. M_r 17 000) and cyanocabalamin (cy, M_r 1350).

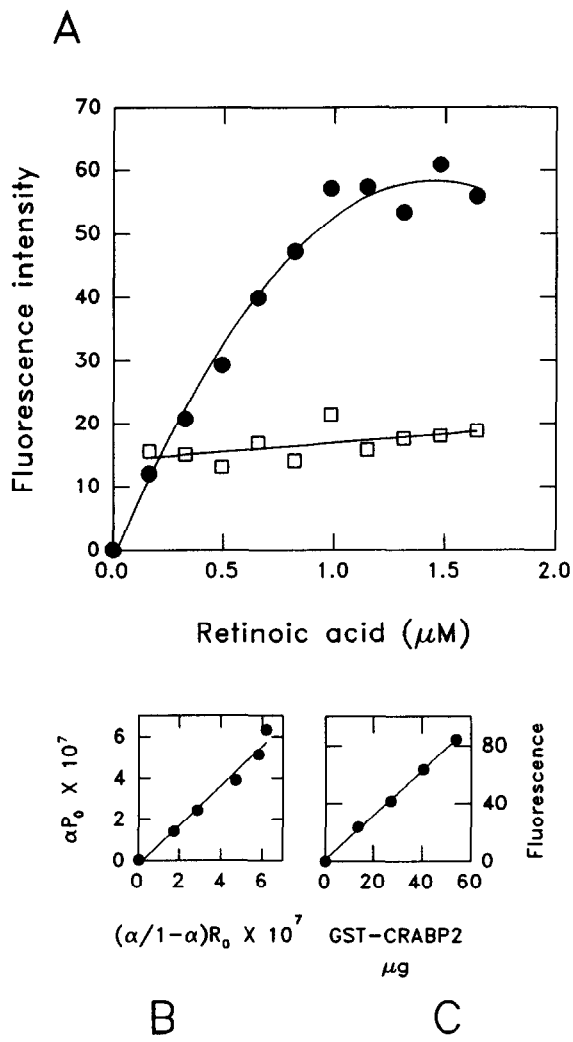

Fig. 4. Fluorimetric titration (350 nm excitation, 475 nm emission) of 0.8 μM GST-CRABP II (●) and GST (□) with *all trans*-retinoic acid. (A) Fluorescence intensity (arbitrary units). To illustrate the trend of the data, 1st and 2rd order regression equations have been fitted to the GST and GST-CRABP II data, respectively. (B) Plot of αP_0 vs. $(\alpha/1-\alpha)R_0$ for the GST-CRABP II data in A. F_{max} is 57.35 and from linear regression, the slope is 0.959, y-intercept is −0.216 and K_d 22.5 nM. (C) Fluorescence intensity after adding increasing amounts of GST-CRABP II to 2 ml of 0.8 μM *all trans*-retinoic acid in 50 mM Tris-HCl, pH 7.5.

in the presence or absence of a 200-fold excess of unlabelled retinoic acid gave a specific binding peak at a position corresponding to the size of the fusion protein (Fig. 3). These data demonstrate that the CRABP II sequence of GST-CRABP II fusion protein specifically binds retinoic acid.

Titration of GST-CRABP II fusion protein with *all trans*-retinoic acid gave increasing fluorescence until the protein was saturated (Fig. 4A). In the presence of excess *all trans*-retinoic acid, fluorescence intensity was directly proportional to the amount of fusion protein added (Fig. 4C). The equilibrium dissociation constant (K_d) was estimated from fluorimetric titrations to be 14.2 ± 6.5 nM (95% confidence limits, n = 11 separate experiments, range 3–36.6 nM). GST protein alone showed no evidence for binding from fluorimetric titration (Fig. 4A). CRABP II cleaved from the fusion protein with thrombin had a similar K_d for retinoic acid to the intact fusion protein (Fig. 5). The effect of thrombin on binding activity was also investigated by incubating

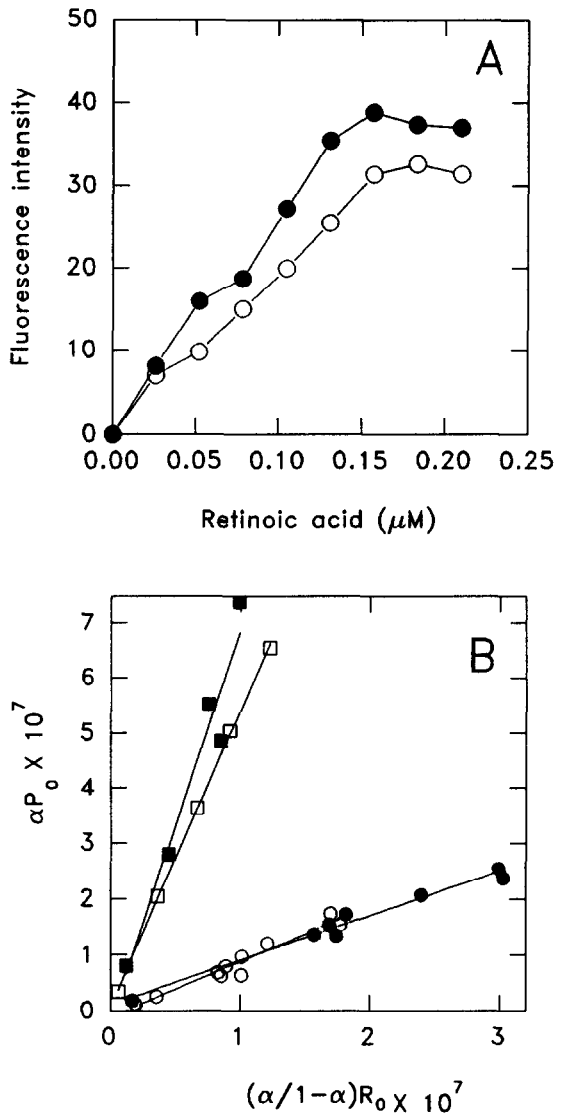

Fig. 5. Fluorimetric titration (350 nm excitation, 475 nm emission) of GST-CRABP II and thrombin-cleaved CRABP II. (A) Fluorescence intensity of 0.5 μM GST-CRABP II cleaved by thrombin (5 U, 1 h at room temperature, ○) and 0.5 μM GST-CRABP II incubated in the absence of thrombin (control, ●) with increasing *all trans*-retinoic acid concentrations. SDS-PAGE analysis of GST-CRABP II after cleavage is shown in Fig. 1, tracks d and e. (B) Plots of αP_0 vs. $(\alpha/1-\alpha)R_0$ for the data in A (□, thrombin-cleaved GST-CRABP II, K_d 1.2 nM; ■, control intact GST-CRABP II, K_d 3.1 nM), and for 0.3 μM GST-CRABP II (●, K_d 10.8 nM) and 0.2 μM purified, thrombin-cleaved CRABP II (Fig. 2) (○, K_d 13.95 nM) titrated against *all trans*-retinoic acid in a separate experiment.

30 μg of GST-CRABP II for 1 h at room temperature with 5 U thrombin in 100 μl buffer C; a parallel, control incubation contained fusion protein but no thrombin. At the end of the incubation, cleavage of fusion protein by thrombin was complete (Fig. 1) and resulted in a slight reduction in binding activity but without any significant change in K_d (Fig. 5).

3.3. *The relative binding of* all trans- *and 9-*cis*-retinoic acid to GST-CRABP II fusion protein*

In initial fluorimetric titration experiments with 9-*cis*-retinoic acid, plots of αP_0 vs. $(\alpha/1-\alpha)R_0$ were curvilinear, suggesting that *cis*→*trans* isomerization was occuring as titration progressed. To avoid this problem, increasing concentrations of either *all trans*- or 9-*cis*-retinoic acid were added to a series of tubes, each containing GST-CRABP II protein, so that the fluorescence intensity of each sample was measured once. These experiments (Fig. 6) showed that, by comparison with *all trans*-retinoic acid, 9-*cis*-retinoic acid bound very poorly to the fusion protein and the extent of binding was too low to obtain a reliable K_d estimate. The binding characteristics of *all trans*-retinoic acid to GST-CRABP II was similar to experiments in which conventional titration was used (Fig. 6).

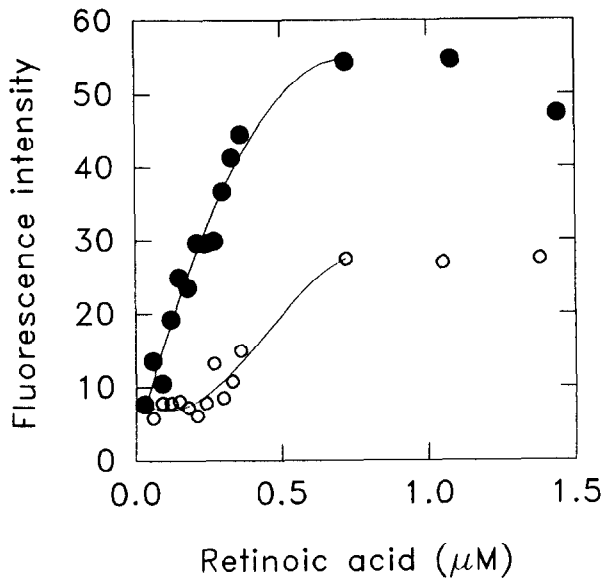

Fig. 6. Fluorimetric titration (350 nm excitation, 475 nm emission) of 0.6 μM GST-CRABP II in the presence of increasing amounts of *all trans*-retinoic acid (●) or 9-*cis*-retinoic acid (○). From plots of αP_0 vs. $(\alpha/1-\alpha)R_0$ (not shown), the K_d for *all trans*-retinoic acid in this experiment was 13.3 nM. For 9-*cis* the K_d estimate was > 600 nM but the extent of binding was too low to obtain a more reliable estimate. Similar results were obtained in two independent experiments.

4. DISCUSSION

CRABP is closely related to a number of other intracellular hydrophobic-ligand binding proteins, including cellular retinol binding protein, myelin P2 protein and fatty-acid binding proteins (FABP). The crystal structure of rat intestinal FABP (I-FABP) and myelin P2 protein has been elucidated [21,22]. These proteins consist of 10 antiparallel β-strands forming a β-barrel structure with two amino-terminal α-helices, and represent a structural paradigm for other members of this family. Arginine residues 112 and 133 of CRABP I protrude into the cavity of the predicted β-barrel and, from structural similarities with ligand–protein interactions in I-FABP [21], may be important in determining ligand specificity [23]. These arginine residues are conserved in CRABP I and II, and the carboxy-terminal amino acid sequences are also very similar [4].

From ligand binding studies, CRABP II has been reported to have a lower affinity for *all trans*-retinoic acid than CRABP I [5,6]. The present data indicate a K_d of 14.2 nM for the binding of *all trans*-retinoic acid to GST-CRABP II. This value is close to the 10 nM reported by Fiorella and Napoli [19] for bovine CRABP I expressed in *E. coli*, an estimate also obtained by fluorimetric titration. The fact that thrombin-cleaved GST-CRABP II had similar binding characteristics to the intact fusion protein suggests that the GST 'leader' sequence does not substantially interfere with the folding of the CRABP II sequence to form a functional and accessible ligand binding pocket. The presence of a glycine rather than a glutamic acid residue at position 71 in the CRABP II coding sequence used to make the GST-CRABP II fusion protein will probably not affect binding affinity: this residue is at the start of the βE-strand [21,22], well away from the ligand binding pocket and predicted entry portal [21], and is orientated away from the β-barrel cavity [22]. Measurements of the affinity of CRABP for retinoic acid have, in the past, produced a range of different values depending on the methodology used [24], and on present results it seems likely that CRABP I and II may have similar affinities for retinoic acid.

9-*cis*-Retinoic acid may be at least as important, if not more so, than *all trans*-retinoic acid in animal development, yet binds very poorly to GST-CRABP II. Similar conclusions have been recently reported by Allenby et al. [25] for the binding of 9-*cis*-[^{3}H]retinoic acid to murine CRABP I and II over-expressed in COS-1 cells. The fact that *all trans*-retinoic acid binds to CRABP with high affinity whereas 9-*cis*-retinoic acid does not implies that there may be other, as yet unidentified, specific binding proteins for 9-*cis*-retinoic acid [2]. Furthermore, CRABP I and II have different patterns of expression [2,3], and this raises the issue of the functional significance of the two forms of CRABP. The ease with which GST-CRABP fusion proteins may be purified from bacterial lysates suggests that they may be powerful tools for comparing the binding affinities of CRABP for different retinoids.

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