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Expression, Purification, and Binding Properties of Human Cellular Retinoic Acid-Binding Protein Type I and Type II[1]

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Human cellular retinoic acid-binding protein (CRABP) type I and type II were expressed in *Escherichia coli* from cloned cDNAs. Expressed proteins were purified by gel filtration and ion-exchange chromatography, resulting in highly pure proteins. The yield after gel filtration was approximately 50 mg/liter bacterial culture. In binding studies the equilibrium dissociation constant, K_d, of retinoic acid (RA) for *E. coli*-derived CRABP-I and CRABP-II was 6.8 and 39 nM, respectively. The K_d of the synthetic retinoid analog CD 367 was 2.2 nM for CRABP-I and 3.0 nM for CRABP-II. RA competed with the binding of CD 367 to CRABP-I and CRABP-II with IC_{50} values of 20.0 and 90.0 nM, respectively. Retinoid analogs competed with the binding of CD 367 to CRABP-I and CRABP-II in the following order: (*p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl)-1-propenyl]-benzoic acid (TTNPB) > 4-*oxo*-RA > 4-OH-RA > 13-*cis*-RA = 9-*cis*-RA. *m*-carboxy-TTNPB and CD 271 were found not to compete with the binding of CD 367 to CRABP-I or CRABP-II even at 500-fold molar excess. These data demonstrate that *E. coli*-derived CRABP-I has a higher affinity for RA than CRABP-II and that retinoic acid metabolites have a lower affinity for these proteins. The observed difference in affinity for RA supports the idea that CRABP-I, which is constitutively expressed, and CRABP-II, which is induced by RA, have different functions in the cell. In addition, 9-*cis*-RA, a natural ligand for the retinoid X receptors, is not a physiological ligand for either CRABP-I or CRABP-II. © 1993 Academic Press, Inc.

Retinoic acid (RA)[3] derived from vitamin A and other retinoids are essential in the control of epithelial cell growth and cellular differentiation (1, 2). Retinoids also play fundamental roles in directing both the spatial organization of cells during development and the generation of vertebrate limbs (3). However, the mechanism of action of retinoids in skin is not completely established. The elucidation of the function of retinoids in these complex biological processes requires identification of the specific components involved in retinoid signal transduction.

Several intracellular retinoic acid-binding proteins and receptors have been identified, the cellular retinoic acid-binding proteins (CRABPs), the nuclear retinoic acid receptors (RARs), and the retinoid X receptors (RXRs) (4–15). RARs have been shown to bind RA with high affinity, whereas RXRs have no affinity for this ligand (12). In contrast, it has recently been shown that 9-*cis*-RA binds to RXR-α with high affinity (16, 17).

We have previously cloned and sequenced human CRABP-I and CRABP-II cDNAs (18) and found a high amino acid homology between human and mouse CRABP-I (99.3%) and human and mouse CRABP-II (93.5%), respectively. In addition, we found that CRABP-II but not CRABP-I mRNA was induced in human skin *in vivo* and in skin fibroblasts *in vitro* by RA (18). CRABP has been detected in a number of different tissues (19, 20) including human (21–24) and rat neonatal skin (25). It has also been shown that CRABP is increased in psoriatic skin compared to normal skin (22, 23). RA has been shown to bind to CRABP with different binding constants (K_d)

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[3] Abbreviations used: RA, retinoic acid; CRABP, cellular retinoic acid-binding protein; RAR, retinoic acid receptor; RXR, retinoid X receptor; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DCC, dextran-coated charcoal; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; TTNPB, (*p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl)-1-propenyl]benzoic acid.

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ranging from 2 to 100 nM (20, 21, 24–26). These differences in affinity of RA for CRABP may result from different assays applied or may be dependent on the source of CRABP. Recently, a K_d of 10 nM was obtained with *Escherichia coli*-derived bovine CRABP-I (27) and a K_d of 65 nM has been reported for neonatal rat CRABP-II (28).

In the present study we have expressed human CRABP-I and CRABP-II in *E. coli,* purified the proteins to homogeneity, and demonstrated that they have different ligand binding properties.

MATERIALS AND METHODS

All-*trans*-retinoic acid, 13-*cis*-retinoic acid, cholesterol, cholic acid, dexamethasone, and thyroid hormone (triiodothyronine) were purchased from Sigma Chemical Co. 4-OH-retinoic acid, 4-*oxo*-retinoic acid, 9-*cis*-retinoic acid, and 1,25-dihydroxyvitamin D_3 were gifts from Hoffman LaRoche (Nutley, NJ). TTNPB (*p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl)-1-propenyl]benzoic acid) and *m*-carboxy-TTNPB (*m*-[(*E*)-2-(5,6,7,8,-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl)-1-propenyl]benzoic acid) were gifts from BASF Aktiengesellschaft (Germany). Linoleic acid and arachidonic acid were purchased from Cayman Chemical (Ann Arbor, MI). [All-*trans*-11,12-^{3}H(N)]retinoic acid with a specific activity of 49.3 Ci/mmol was purchased from New England Nuclear. [^{3}H]CD 367 (4-[5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl]benzoic acid) with a specific activity of 52.8 Ci/mmol, nonradioactive CD 367, and CD 271 (6-[3-(1-adamantyl)-4-methoxyphenyl]-2-naphthoic acid) were gifts from CIRD (Valbonne, France). The pET Expression system was purchased from Novagen (Madison, WI).

Cloning and expression of CRABP-I and CRABP-II in E. coli. cDNAs for human CRABP-I and CRABP-II (18) were cloned into the unique *Nde*I and *Bam*HI sites of the expression vector pET-3a. Briefly, an *Nde*I site was introduced at the 5′ end of the CRABP-I and CRABP-II cDNAs using synthetic oligonucleotides and the polymerase chain reaction (PCR). The cDNAs were subsequently cloned into the pET-3a vector, such that transcription of the entire coding region would be initiated at the ATG translation initiation codon. The PCR-derived constructs were confirmed by sequencing. The plasmids (pET-3a/CRABP-I or pET-3a/CRABP-II) were used to transform bacteria (BL21(DE3)/pLysS)(Novagen). Transformed bacteria were plated onto agar plates containing ampicillin (50 μg/ml). Single colonies were used to inoculate an overnight culture of 10 ml YT medium containing ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml). Three hundred microliters from the overnight culture was used to inoculate 2-liter flasks with 300 ml YT medium containing ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml). Bacteria were grown to an OD_{595} of 0.6. Isopropyl β-D-thiogalactopyranoside (IPTG, final concentration 0.6 mM) was then added and bacteria were grown for an additional 2 h at 37°C.

Purification of CRABP-I and CRABP-II protein by FPLC. Bacteria were pelleted by centrifugation (5000*g* for 20 min). To induce lysis of bacteria, samples were frozen at −70°C and thawed three times. Lysed bacteria were then treated as described (27). The 10,000*g* supernatant was applied to a Sephadex FPLC column and eluted isocratically at 3 ml/min with 125 mM NaCl, 50 mM bis-Tris-HCl (pH 7.0), and 2 mM β-mercaptoethanol. Fractions of 2 ml were collected and analyzed by SDS–PAGE. The fractions containing CRABP-I or CRABP-II were collected, pooled, and further analyzed by FPLC ion-exchange using a Mono-Q column equilibrated with 20 mM bis-Tris–HCl (pH 7.0) and 2 mM β-mercaptoethanol as described (27). CRABP-I and CRABP-II were eluted at 1 ml/min using a linear gradient of NaCl in the same buffer from 0 to 0.5 M.

Ligand-binding assay. For binding of retinoids and other compounds we used the dextran-coated charcoal (DCC) assay (25). Briefly, 1-ml aliquots of CRABP-I or CRABP-II protein were incubated overnight at 4°C with ^{3}H-labeled ligand in the presence of at least 100 times molar excess unlabeled ligand. For competition assays ^{3}H-CD 367, a chemically stable retinoid analog, was used at 2 nM concentration. DCC was added to separate bound from free ligand and samples were centrifuged. Scintillation fluid was then added to the supernatant and samples were counted for 4 min in a Beckman LC-5801 liquid scintillation counter. Equilibrium dissociation constants, K_d, of the compounds tested were determined by Scatchard analysis.

RESULTS AND DISCUSSION

Cloning and expression of CRABP-I and CRABP-II in E. coli. CRABP-I or CRABP-II cDNAs were modified using synthetic oligonucleotides and PCR to introduce an *Nde*I site at the ATG translation initiation codon. The complete coding regions of the CRABP-I and CRABP-II cDNAs were subsequently cloned downstream from the T7 promoter of the plasmid pET-3a. The T7 promoter is controlled by the lac UV5 promoter, which in turn is activated by IPTG (27). Once IPTG is added to the medium, bacteria are induced to produce CRABP protein from the cDNAs. No 15-kDa protein was observed in uninduced cultures (i.e., cultures without IPTG added), whereas a time-dependent increase in the formation of a 15-kDa protein was seen for both CRABP-I and CRABP-II. The formation of protein peaked at 2 h after induction (as determined by SDS–PAGE, data not shown).

Purification of CRABP-I and CRABP-II protein by FPLC. Bacteria were harvested by centrifugation followed by lysis. Aliquots of the 10,000*g* supernatant of the lysed bacteria were analyzed by SDS–PAGE (Fig. 1, lanes 1 and 2). The major protein band obtained after expression had the predicted molecular weight of CRABP-I and CRABP-II (approximately 15 kDa) in both cases. The 10,000*g* supernatants were subjected to FPLC gel filtra-

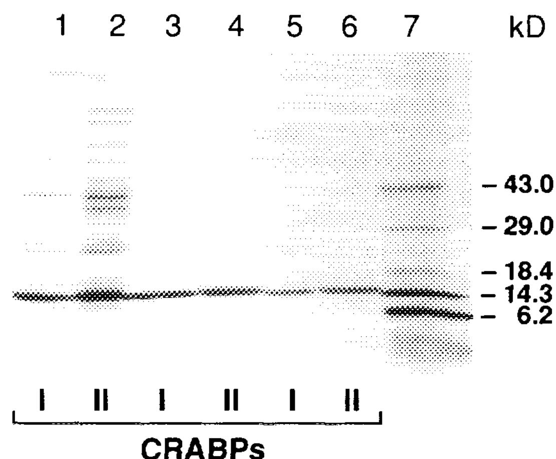

FIG. 1. SDS–PAGE analysis of CRABP-I and CRABP-II expressed in *E. coli.* The 10,000*g* supernatants (lanes 1 and 2), gel filtration fractions (lanes 3 and 4), and ion-exchange fractions (lanes 5 and 6) were fractionated on a 4–20% SDS-polyacrylamide gel and stained with Coomassie blue. Lane 7 represents molecular weight standards.

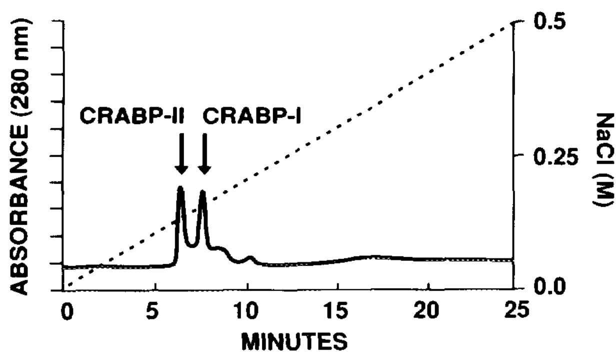

FIG. 2. FPLC ion-exchange chromatogram of FPLC gel filtration purified CRABP-I and CRABP-II (100 μg of each protein). Flow rate: 1 ml/min. A linear gradient of NaCl from 0 to 0.5 M was applied.

tion. The presence of CRABP was determined by analyzing successive fractions eluting from the gel filtration column by SDS–PAGE (data not shown). Both CRABP-I and CRABP-II eluted with the same retention time from the column. Fractions of 15 kDa were pooled and analyzed for purity by SDS–PAGE (Fig. 1, lanes 3 and 4). Only a 15-kDa band appeared on the gel after staining, indicating that the CRABP proteins were pure after gel filtration. The yield of CRABP at this stage was approximately 50 mg/liter of bacterial culture. To test the purity of the produced proteins, pooled gel filtration fractions were analyzed by ion-exchange chromatography. A single peak appeared in the chromatograms, further supporting the purity of CRABP protein (data not shown). CRABP-I eluted at a NaCl concentration of 150 mM, whereas CRABP-II eluted at 125 mM NaCl. In the study of Fiorella and Napoli (27) an additional peak was seen in the ion-exchange chromatograms. No such additional peak was observed in our chromatograms. Fractions eluting from the ion-exchange column were also run on an SDS–PAGE gel (Fig. 1, lanes 5 and 6). No other band than the CRABP-I and CRABP-II bands could be detected.

In order to determine whether CRABP-I could be separated from CRABP-II by use of FPLC ion exchange, 100 μg of each protein from the gel filtration pool was applied to the ion-exchange column (Fig. 2). CRABP-I was clearly separated from CRABP-II by 1 min and baseline was reached between each protein. This provides a method for the separation of human CRABP-I and CRABP-II in biological tissues and opens the way for further analysis of the role of CRABP in biological processes.

Binding of RA to CRABP-I and CRABP-II. A typical curve for the binding of RA to CRABP-I is shown in Fig. 3A. Scatchard analysis of the binding data (Fig. 3A, inset) gave a K_d value of 6.8 nM, which is in the same range as previously reported (27). Higher concentrations of RA were required to obtain saturation of CRABP-II (Fig. 3B), and a K_d of 39 nM was obtained by Scatchard analysis (Fig. 3B, inset). These data show that CRABP-I has a 5.7 times higher affinity for RA than CRABP-II. Very recently the affinity of RA for CRABP-I and CRABP-II was determined in human skin using a PAGE/autoradioblotting technique (29). It was reported that CRABP-I (K_d = 16.6 nM) had a higher affinity for RA compared to CRABP-II (K_d = 50 nM), which is in close agreement with the data obtained in the present study using *E. coli*-derived CRABPs. Increased levels of CRABP have previously been found in lesional psoriatic skin compared to uninvolved skin (22, 23), and it has been shown that lesional psoriatic skin has an increased CRABP-binding capacity for RA (21). It was recently shown that this increase was due to a higher level of CRABP-II protein in psoriatic skin (29). We have previously shown that CRABP-II but not CRABP-I is induced in human skin *in vivo* and in skin fibroblasts *in vitro* by RA (18). It has also recently been shown that CRABP-II mRNA is selectively increased in psoriatic skin compared to normal skin (23, 30). It is possible that CRABP-I, which is constitutively expressed, and CRABP-II, which is induced by RA, have different functions in the cell. CRABP-II

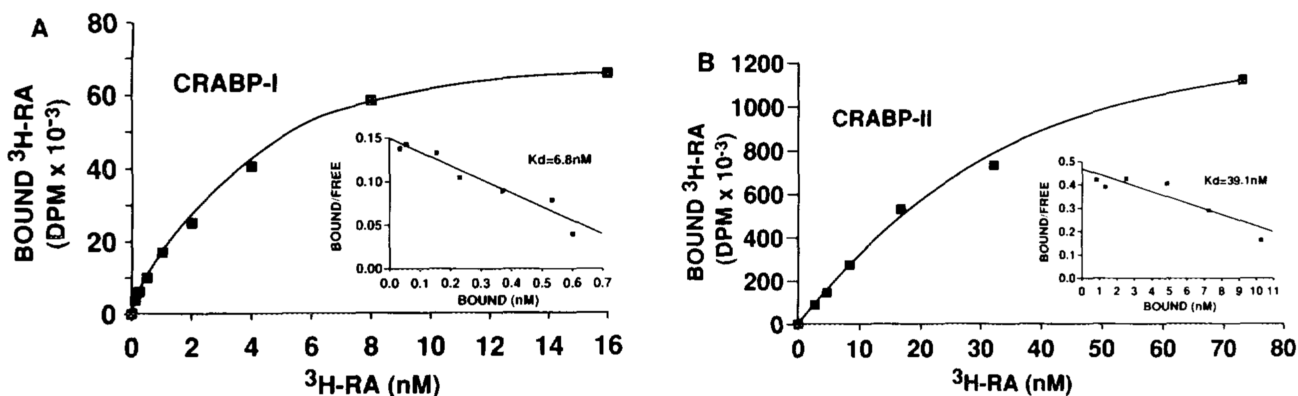

FIG. 3. Binding of retinoic acid to CRABP-I (A) and CRABP-II (B). Insets: The equilibrium dissociation constant (K_d) was determined by Scatchard analysis of the binding data.

TABLE I

Competition of Different Retinoids and Retinoid Analogs with the Binding of ^{3}H-CD 367 to Purified CRABP-I and CRABP-II Expressed in *E. coli*

Competitor	CRABP-I	CRABP-II
	IC_{50} (nM)	
CD 367	16	20
All-*trans*-RA	20	90
TTNPB	160	40
4-*oxo*-RA	300	500
4-OH-RA	>1000	NC[a]
13-*cis*-RA	>1000	>1000
9-*cis*-RA	>1000	>1000
m-carboxy-TTNPB	NC[a]	NC[a]
CD 271	NC[a]	NC[a]

[a] NC, no competition with concentrations up to 1 μM. Compounds were tested in the concentration range 2.5 mM–1 μM.

could act as a buffer for RA, since CRABP-II mRNA is induced by RA at concentrations as low as 0.3 nM, reaching a maximum between 300 and 3000 nM (31). In agreement with this hypothesis is the lower affinity of RA for CRABP-II (39 nM) compared to CRABP-I (6.8 nM), which would allow the presence of small amounts of free RA in the cell while excess amounts would be bound by the RA-inducible CRABP-II.

Competition for binding of ^{3}H-CD 367 to CRABP-I and CRABP-II by retinoids. The retinoid analog ^{3}H-CD 367 was used in competition studies instead of RA, since it is chemically stable and was found to have high affinity for both CRABP-I and CRABP-II. Scatchard analysis of binding curves obtained using ^{3}H-CD 367 (data not shown) gave K_d values of 2.2 nM for CRABP-I and 3.0 nM for CRABP-II. Consequently, we used the competition assay to determine the ability of different retinoid-like compounds to compete with the binding of ^{3}H-CD 367 (2 nM) to CRABP-I and CRABP-II, (Table I). As can be seen, TTNPB and 4-*oxo*-RA were able to complete with the binding of ^{3}H-CD 367 to CRABP-I and CRABP-II, while 4-OH-RA, 13-*cis*-RA, and 9-*cis*-RA demonstrated a weak competition with an IC_{50} value above 1000 nM. CD 271 and *m*-carboxy-TTNPB did not compete when concentrations up to 1000 nM were used (Table I). The lack of binding of CD 271 to CRABP is in accordance with the results obtained when cytosolic extracts from human cultured fibroblasts and keratinocytes were used (32). TTNPB demonstrated a higher affinity for CRABP-II than CRABP-I. 4-*oxo*-RA demonstrated a similar affinity for both CRABPs, while 4-OH-RA had a very low affinity for both proteins. The RA isomers 9-*cis*-RA and 13-*cis*-RA demonstrated a very low affinity for both CRABP-I and CRABP-II. It has been shown that the RARs have high affinity for RA, while the RXRs apparently do not bind this compound (12). In contrast it has recently been demonstrated that 9-*cis*-RA binds with high affinity to RXR-α (16, 17). Our data clearly demonstrate that 9-*cis*-RA is not a physiological ligand for CRABP-I and CRABP-II. Nonretinoids such as linoleic acid, arachidonic acid, cholic acid, cholesterol, dexamethasone, 1,25-dihydroxyvitamin D_3, and thyroid hormone (T_3) were found not to compete with the binding of ^{3}H-CD 367 to either CRABP-I or CRABP-II (data not shown).

The observed difference in the ability of CRABP-I and CRABP-II to bind RA further supports that these two proteins indeed may have different physiological functions. However, what these functions may be remains to be established.

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