Vol. 155, No. 1, 1988
August 30, 1988

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 503-508

AFFINITY GELS FOR PURIFICATION OF RETINOID-SPECIFIC BINDING PROTEIN (RSBP)

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Received June 20, 1988

Retinoids are defined as compounds which elicit specific biological effects such as control of cell growth and cell differentiation by binding to a specific receptor. Recently, we demonstrated the presence of a protein (RSBP) which satisfies the criteria for the retinoid receptor. For purification of RSBP, we prepared two types of affinity gels with retinoidal ligands (Gel-Am and Gel-Ch) based on synthetic retinobenzoic acids which possess very potent retinoidal activities. RSBP in the crude fraction extracted from cultured cells could be purified about 300-fold by affinity column chromatography using these affinity gels.

Retinoids have been drawing much attention as agents controlling cell growth and cell differentiation.[1,2] It is believed that retinoids elicit the specific biological effects (retinoidal activities) by binding to a specific receptor,[1-4] though the molecular mechanisms of the retinoidal activities are not well understood. A classical ligand for such a retinoid receptor is all-trans-retinoic acid (RA), and many synthetic retinoids have been reported.[1,2] Recently, we reported the synthesis, biological effects and structure-activity relationships of a new structural class of retinoids, retinobenzoic acids.[5-8] Among the synthesized retinobenzoic acids, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)benzoic acid (Am80)[5] and (E)-4-[3-(3,5-di-tert-butylphenyl)-3-oxo-1-propenyl]benzoic acid (Ch55)[6] possess quite potent retinoidal activities. These compounds should also elicit their biological effects by binding to the retinoid receptor.[3,4] One of the candidates for such a retinoid receptor has been CRABP.[1,2] However, Am80 and Ch55 (which show more potent bioactivities than RA in various biological assay systems) bind to CRABP with much lower affinity

0006-291X/88 $1.50

than RA[9-11]; the affinities of Am80 and Ch55 for CRABP were estimated to be 1/80 and 1/530 of that of RA, respectively.[9] The true retinoid receptor should bind to all the compounds which possess retinoidal activity[4].

Very recently, we demonstrated the presence of a protein in human promyelocytic leukemia cell line HL-60 (which possesses high response to retinoids, and which is reported to possess no detectable amount of CRABP[9,12,13]) that binds to RA, Am80 and Ch55 with very high affinity in a mutually competitive manner.[14] This protein satisfies the criteria for the true retinoid receptor, and was named retinoid-specific binding protein (RSBP).[14] RSBP exists predominantly in the nuclear fraction, and is a protein with an apparent molecular weight of 95 kDa, possessing very high binding affinity for RA, Am80 and Ch55 (association constants toward these retinoids are of the order of 10^{10} M^{-1}).[14]

On the other hand, the groups of Chambon[15,16] and Evans[17] reported a cloning of retinoic acid receptor gene on the basis of the assumption that the retinoic acid receptor should resemble steroid receptors. The molecular weights of the products of their cloned genes were reported to be around 50 kDa.[15-17] To investigate the relation between these products of cloned genes and RSBP, and to reveal the molecular mechanism of retinoidal action, it is essential to isolate and to characterize RSBP.

In this paper, we describe the synthesis of affinity gels (Figs. 1 and 2) based on new-type retinobenzoic acids, and the partial purification of RSBP by use of the prepared affinity gels.

Materials and Methods

Synthesis of the Affinity Ligand 3 (Fig. 1):

5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthoic acid (1, 1.512 g) was converted to the acid chloride by thionyl chloride treatment, and was condensed with methyl p-aminosalicylate (1.08 g) in the presence of a catalytic amount of 4-dimethylaminopyridine in a mixture of dry benzene (30 ml) and pyridine (3 ml) to give the amide compound 2 in the yield of 64.6% (11.6 g, mp. 227.5-228.0 ℃). The compound 2 (0.5 g) was treated with sodium hydride (NaH) in dry dimethylformamide(5 ml), and then mixed with $BrCH_2CH_2CH_2NHCOOCH_2Ph$ (0.44 g). The mixture was stirred at room temperature for 15 hr. The protected affinity ligand thus obtained (yield: 50.8%) was deprotected with potassium hydroxide (hydrolysis of the ester group, yield: 93.5%) and then with trifluoroacetic acid (CF_3COOH; debenzyloxycarbonylation) to give affinity ligand 3 in the yield of 68.1%.

Synthesis of the Affinity Ligand 7 (Fig. 1):

5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthylmethylketone (4, 36.8 g) was heated with m-chloroperbenzoic acid (mCPBA, 45 g) in chloroform (200 ml) for several hours to give compound 4 in the yield of 87.6% (34.5 g). Compound 5 was heated at 130-140 ℃ with aluminum chloride ($AlCl_3$, 37 g) to give acetyl-rearranged product, 5,6,7,8-tetrahydro-3-hydroxy-5,5,8,8-tetra-

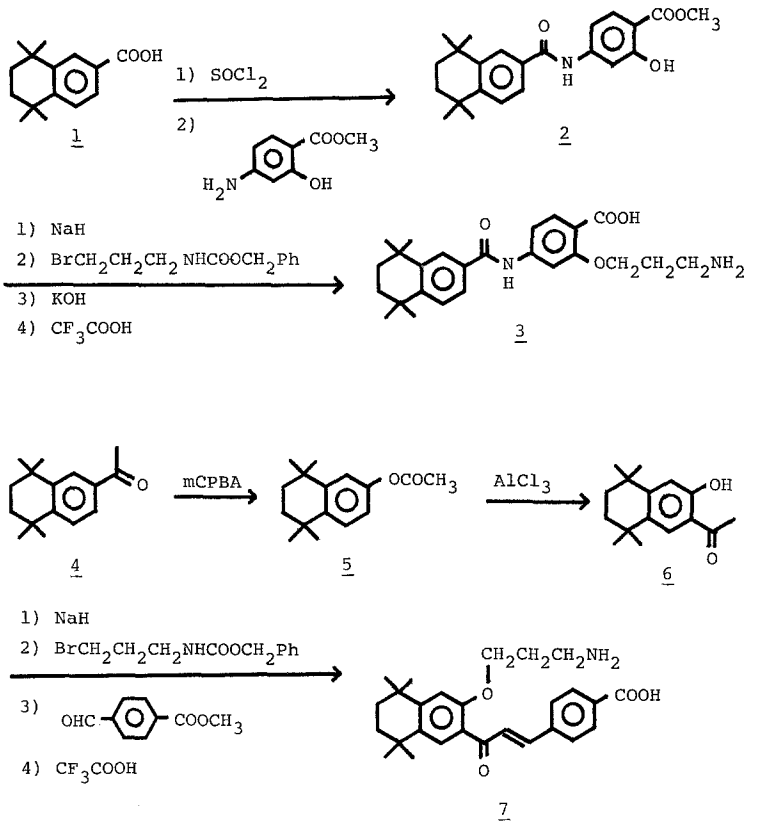

Fig. 1. Synthesis of affinity ligands 3 and 7

methyl-2-naphthylmethylketone (6) in the yield of 77.7% (48.2 g). The hydroxyl group of 6 (2.532 g) was alkylated with $BrCH_2CH_2CH_2$-$NHCOOCH_2Ph$ by the method described above to give the ortho-alkoxy ketone in the yield of 83.6% (3.70 g). The aldol condensation of this ketone(2.0 g) with terephthalaldehydic acid methyl ester (0.75 g) in a mixture of 2 N sodium hydroxide (5 ml) and ethanol (30 ml) gave protected affinity ligand 7, which was deprotected by refluxing in CF_3COOH to give affinity ligand 7.

Preparation of the Affinity Gels Am and Ch (Fig.2):

The N-hydroxysuccinimide ester of agarose (Affi-Gel 10) was obtained from Bio-Rad Laboratories. The gel (2 ml in isopropanol) was washed three times with 2 ml of dioxane and was resuspended in 2 ml of dioxane. To this suspension, ligand 3 or ligand 7, prepared as described above, and triethylamine (12.5 μ l) were added at 25 ℃ and reacted for 2 hr. Then the unreacted N-hydroxysuccinimide ester was blocked by treatment with 0.1 ml of 1 M ethanolamine for 1 hr. The mixture was diluted by addition of dioxane (6 ml), and centrifuged (2000 rpm x 4 min), and then decanted. The gel was washed successively with dioxane, ethanol and phosphate buffered saline (PBS; 136.9 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$), and stocked in PBS containing 0.02% sodium azide at 4 ℃. The coupling efficiencies were estimated from UV absorption measurements.

Extraction of RSBP from Cultured HL-60 Cells:

HL-60 cells were cultured, collected and washed as described previously.[14] The cell pellet was homogenized and fractionated as described previously[14] except that the homogeniz-

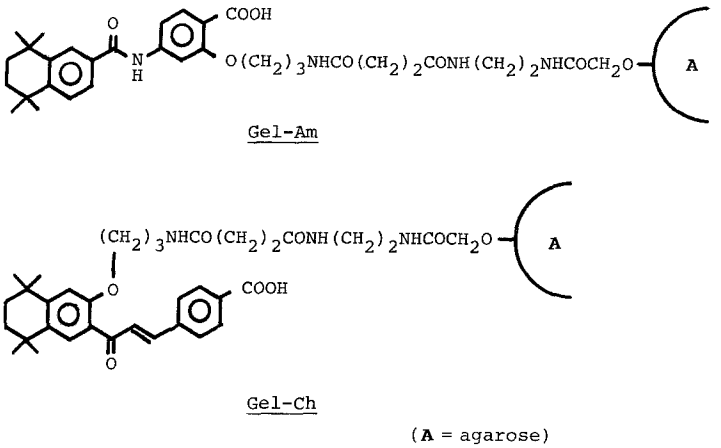

Fig. 2. Structures of affinity gels, Gel-Am and Gel-Ch

ing buffer was changed to PBS containing 20 mM Tris (pH 8.0), 0.6 M KCl, 1 mM dithiothreitol (DTT), 20 mM molybdate (Na_2MoO_4) and a protease inhibitor cocktail (PIC: 1 mM phenylmethylmethanesulfonyl fluoride, 0.1 mg/ml bacitracin, 0.1 mM leupeptin, 1μ g/ml pepstatin, 0.1 mg/ml aprotinin and 1.5 mM EDTA).

The amount of protein was estimated by the Coomassie blue method as described by Bradford.[18]

The amount of RSBP was estimated by filter binding assay of the samples incubated with tritium-labeled retinoids [^{3}H-Am80 (65 Ci/mmole), ^{3}H-Ch55 (20 Ci/mmole) or ^{3}H-RA (50 Ci/mmole)] in the presence or absence of excess amounts of cold retinoids (Am80, Ch55 or RA) at 4℃ as described previously.[14] ^{3}H-Am80 and ^{3}H-Ch-55 were prepared by Amersham. ^{3}H-RA was purchased from NEN Research Products.

Partial Purification of RSBP Using Affinity Gel Am or Ch

Affinity gel Am or Ch (2 ml) was packed in a glass tube (0.7 x 10 cm) and equilibrated with washing buffer (20 mM Tris pH 8, 0.3 M NaCl, 1 mM DTT). A crude soluble protein fraction containing RSBP extracted from cultured HL-60 cells as described above was applied on the packed affinity gel column with a flow rate of 9 ml/h. Then the column was washed with 50 ml of the washing buffer (9 ml/h). RSBP absorbed on the gel was eluted with elution buffer (20 mM Tris pH8, 1 mM DTT) alone or the elution buffer containing 20 mM Am80 (9 ml/h). The eluent was applied on a Mono Q HR5/5 column (obtained from Pharmacia) and eluted with a linear gradient of 0-0.5 M NaCl in the elution buffer as described previously.[14] RSBP was eluted with 0.20-0.25 M NaCl in the elution buffer. The amounts of protein and RSBP were estimated by the Coomassie blue method as described by Bradford[18] and by filter binding assay,[14] respectively.

Results and Discussion

It is essential to isolate and characterize the retinoid receptor to elucidate the molecular mechanism of retinoidal action. As we had established the presence of RSBP, which satisfies the criteria for the retinoid receptor in HL-60 cells,[14] we started to purify RSBP. For this purpose, we designed the

affinity gels with retinoidal ligands (Gel-Am and Gel-Ch) as shown in Fig.2.

Fig. 1 briefly shows the synthetic routes employed to obtain the affinity ligands 3 and 7. These ligands were attached to agarose by coupling with N-hydroxysuccinimide ester of agarose (Affi-Gel 10, Bio-Rad Laboratories). From the UV absorption of the gels, it was estimated that 5-10μ mole of the ligands was attached to 1 ml of the gel.

Both the prepared gels (Gel-Am and Gel-Ch) possess quite similar properties. They absorbed RSBP very efficiently when the pH was higher than 7.4, and the salt concentration was rather high (more than 0.3 M for NaCl). One ml of the gels was sufficient to absorb 360 pmole of RSBP under the conditions described in the Materials and Methods. When the pH was adjusted to below 7.2, the ability of the gel to absorb RSBP was diminished; at pH 6.8, RSBP was recovered almost quantitatively in the fraction that passed through the column without absorption. This suggests that the ionization of the retinoidal ligands to the corresponding carboxylate anion form is essential to bind to RSBP. In addition, low ionic strength also diminished the binding efficiency of the gels to RSBP, which suggests that there is a hydrophobic interaction between the retinoidal ligands and RSBP, overwhelming the ionic interaction.

The absorbed RSBP could be eluted with low salt buffer (elution buffer) alone, though the elution efficiency was low; 150 ml of the elution buffer was needed to elute 60 pmole of RSBP absorbed on 2 ml of the gels. Though the recovery of the retinoid-binding activity was not excellent (25-30%, probably because of denaturation under the very dilute conditions), RSBP was purified about 300-fold as shown in Table I.

The RSBP absorbed on the gels was also eluted with elution buffer containing 20 mM Am80 (less than 30 ml was enough to elute the RSBP absorbed on 2 ml of the gels). Eluted RSBP-Am80 complex was separated on a Mono Q HR5/5 column (Pharmacia) as described in the Materials and Methods. The chromatogram showed only one major peak at the retention time of RSBP-Am80 complex (data not shown).[14] The pooled fraction was incubated with ^{3}H-Am80 or ^{3}H-Ch55 in the presence or absence of an excess amount of Ch55. The ligand exchange reaction is very slow at 4 ℃, and it took more than 72 hr for the specific binding to reach a plateau. By affinity column (Gel-Am) and Mono Q chromatography, RSBP was purified more than 4000-fold with the total recovery of the retinoid-binding activity of about 8% from the crude soluble protein fraction (Table I). A quantitative consideration suggests

Table I. Purification of RSBP in Crude Soluble Protein Fraction Extracted from HL-60 Cells

Columns	Loaded Sample		Eluted Fraction			Purification (-fold)
	Protein (mg)	RSBP (pmole)	Protein (mg)	RSBP (pmole)	Yield (%)	
Gel-Am[a]	106.0	21.20	0.112	6.36	30	284
Gel-Ch[a]	99.0	17.82	0.078	4.63	26	329
Gel-Am[b] / Mono Q	102.0	14.79	0.002	1.26	8.5	4345
Gel-Ch[b] / Mono Q	104.0	15.20	0.001	1.18	7.8	8108

a) RSBP absorbed on the gels was eluted with elution buffer alone.
b) RSBP absorbed on the gel was eluted with elution buffer containing 20 mM Am80, and then separated on a Mono Q column.

that another step of 10- to 50-fold purification would be sufficient for the purification of RSBP to homogeneity.

In conclusion, affinity gels for the purification of RSBP were designed and prepared. The gels were confirmed to be effective for the purification of RSBP. By the use of the prepared affinity gels and a Mono Q column, RSBP could be purified 4000- to 8000-fold.

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