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Specific binding of [^{3}H]retinoids to cellular retinoid-binding proteins

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The specific binding of [^{3}H]retinoids to cellular retinoid-binding proteins was measured directly by the cold acetone filtration method. After incubation of purified cellular retinoid-binding proteins with [^{3}H]retinoids with or without competitors for 2–4 h, bound ligands were separated from free by filtration using cold acetone. Nonspecific binding of the ligands was reduced sufficiently to allow measurement of specific binding of [^{3}H]retinoids to cellular retinoid-binding proteins. This method has the advantages of being rapid and practical and giving reproducible results.

Retinol-binding protein; Retinoic acid-binding protein; Retinoid binding

1. INTRODUCTION

The intracellular transport vehicles of retinoids are believed mainly to be CRBP and CRABP [1]. The binding of retinoids to cellular retinoid-binding proteins has been studied by competitive binding experiments using sucrose gradient centrifugation [2–6], gel filtration [7], polyacrylamide disc gel electrophoresis [8] or charcoal assay [9]. However, the separation of bound and free ligands by these procedures is time-consuming. Recently, HPLC has been used to achieve more rapid separation [10,11]. However, even more rapid separation is required to avoid the possibility that the equilibrium of the proteins and ligands changes during separation. Moreover, in previous experiments, crude or only partially purified cellular retinoid-binding proteins were used, and nonspecific binding components, such as albumin, were not removed completely.

Here, we established a rapid method for direct measurement of specific binding of retinoids to purified cellular retinoid-binding proteins. Ashendel et al. reported direct measurement of specific binding of highly lipophilic 12-*O*-[^{3}H]tetradecanoylphorbol 13-acetate to a mouse skin particulate fraction using cold acetone [12]. We applied this cold acetone filtration method for assay of specific binding of retinoids to purified cellular retinoid-binding proteins. It was possible to separate protein-bound and free ligands simultaneously, and to demonstrate the specific binding of [^{3}H]retinoids to purified CRBP or CRABP. We used this method to study the binding affinity and ligand specificity. This cold acetone filtration method should be useful for elucidating the functions of cellular retinoid-binding proteins.

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Abbreviations: CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; HPLC, high-performance liquid chromatography; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Materials*

all-*trans*-Retinol and all-*trans*-retinoic acid were purchased

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from Sigma (St. Louis, MO). all-*trans*-[15-^{3}H]Retinol (20 Ci/mmol) and all-*trans*-[11,12-^{3}H]retinoic acid (46.7 Ci/mmol) were obtained from New England Nuclear (Boston, MA). The purity of retinoids was determined by thin-layer chromatography with toluene/chloroform/methanol (4:1:1) as solvent. Lysozyme was from Tokyo Seikagaku Kogyo. Bovine adrenal glands were purchased from Tokyo Shibaura Zoki.

2.2. *Binding assay*

Specific binding of [^{3}H]retinol or [^{3}H]retinoic acid to 1–10 μg purified CRBP or CRABP was measured in 1 ml incubation buffer (50 mM Tris-HCl buffer, pH 7.4, containing 2 mM 2-mercaptoethanol), in silanized glass test tubes (13 × 100 mm). [^{3}H]Retinoic acid with a final specific activity of 20 Ci/mmol was prepared from labeled and unlabeled compounds. Labeled compound dissolved in 2 μl dimethyl sulfoxide (DMSO) was added to each sample to give the final concentration specified. Total binding was determined with 3 μl DMSO. Nonspecific binding was determined with a 200-fold excess of nonradioactive all-*trans*-retinol or all-*trans*-retinoic acid. Competitors were added at various concentrations in binding inhibition studies. Incubation was carried out at 4°C for 2–4 h under dim light with gentle shaking. After incubation, 100 μg lysozyme in 100 μl incubation buffer was added as a coprecipitant. Then 2.5 ml acetone that had been cooled in a dry ice-ethanol bath (−78°C) was promptly added to the reaction mixture with vigorous mixing with a vortex mixer for a few seconds. The mixture was filtered through a 2.4 cm diameter Whatman GF/C glass microfiber filter, with aspiration. The tube and the filter were washed 4 times with 2.5 ml cold acetone, and the radioactivity remaining on the filter was counted in a liquid scintillation counter. Every assay was carried out in duplicate. Specific binding was calculated as the difference between the averages of the total binding and nonspecific binding.

2.3. *Purification of CRBP and CRABP*

CRBP and CRABP were purified from 550 bovine adrenal glands (total 6.6 kg) by the procedures reported from this laboratory [13]. The fractions containing CRBP and CRABP were monitored by measurement of fluorescence (excitation at 350 nm, emission at 480 nm) and by SDS-PAGE. The fluorescence of CRBP was due to protein-bound endogenous retinol, and that of CRABP to protein-bound authentic retinoic acid. The tissue was homogenized, precipitated with acid and treated batchwise with CM-cellulose (CM52, Whatman), and the resulting solution was chromatographed sequentially on DEAE-cellulose (DE52, Whatman), Sephadex G-50 (Pharmacia) and hydroxyapatite (Bio-Gel HT, Bio-Rad). Totals of 14.7 mg CRBP and 28.2 mg CRABP were purified to homogeneity. Each protein appeared to be a single polypeptide with a molecular mass of 16 kDa on SDS-PAGE.

2.4. *SDS-PAGE*

The molecular masses and purities of cellular retinoid-binding proteins were determined by electrophoresis on 15% polyacrylamide slab gels containing SDS [14].

2.5. *Determination of protein*

The amount of protein was measured by the method of Bradford [15].

3. RESULTS AND DISCUSSION

3.1. *Direct measurement of the specific binding of [^{3}H]retinol or [^{3}H]retinoic acid to cellular retinoid-binding proteins*

Fig.1A shows that specific binding of [^{3}H]retinol to CRBP was linearly proportional to the amount of protein. Fig.1B shows that the specific binding of [^{3}H]retinol to CRBP was saturable. A Scatchard plot of the equilibrium binding data indicated that a single class of binding sites was the most likely and that the dissociation constant (K_d) was 5.5×10^{-7} M (fig.1B). The maximum binding capacity (B_{max}) was 8.0 nmol/mg CRBP. The specific binding of [^{3}H]retinoic acid to CRABP also demonstrated linearity and saturation (fig.2). A Scatchard plot indicated that there was probably a single class of binding sites of CRABP for retinoic acid with a K_d of 6.9×10^{-8} M and B_{max} of 2.2 nmol/mg CRABP (fig.2B). Thus, the specific binding of retinoids to purified cellular retinoid-binding proteins could be clearly demonstrated by the cold acetone filtration method. The nonspecific binding of [^{3}H]retinol and [^{3}H]retinoic acid to the purified cellular retinoid-binding proteins was sufficiently low to allow measurement of specific binding by the cold acetone filtration method. The dissociation constants were higher than those of purified rat CRBP and CRABP determined by fluorimetric titration [16,17]. This difference may be due to the different methods used for their determination and the various sources of cellular retinoid-binding proteins.

3.2. *Binding inhibition studies*

The specific binding of [^{3}H]retinol to CRBP was completely inhibited by nonradioactive retinol. From the competition curve (fig.3A), the concentration of retinol for 50% inhibition was determined to be about 100 nM. Nonradioactive retinoic acid did not inhibit binding at concentrations below 100 nM, however at concentrations higher than 1 μM, it inhibited the specific binding of [^{3}H]retinol to CRBP (about 35% inhibition at 10 μM). Until now it has been believed that a free alcohol in the C-15 position of retinoids is required for binding to CRBP and a free carboxyl group at the C-15 position is necessary for binding to CRABP [4,6,9,18]. However, our results suggest that at high concentrations, retinoic acid binds to

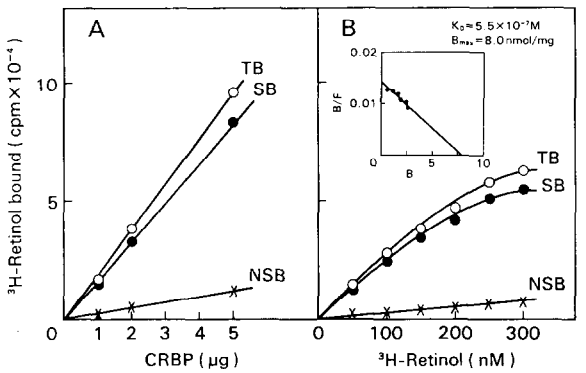

Fig.1. Specific binding of [^{3}H]retinol to CRBP. Various concentrations of CRBP were incubated with a fixed concentration of 50 nM [^{3}H]retinol (A) and 1 μg CRBP was incubated with various concentrations of [^{3}H]retinol (B). Total bound [^{3}H]-retinol (TB,○), specifically bound [^{3}H]retinol (SB,●) and non-specifically bound [^{3}H]retinol (NSB,X) were measured as described in section 2. The inset in B shows a Scatchard plot of the data [19], where B represents the amount (nmol/mg protein) of specifically bound [^{3}H]retinol and F the concentration (nM) of free [^{3}H]retinol. The dissociation constant (K_d) of [^{3}H]-retinol from CRBP was estimated to be 5.5×10^{-7} M and the maximum binding capacity (B_{max}) of [^{3}H]retinol to CRBP to be 8.0 nmol/mg CRBP. Points are means for duplicate determinations.

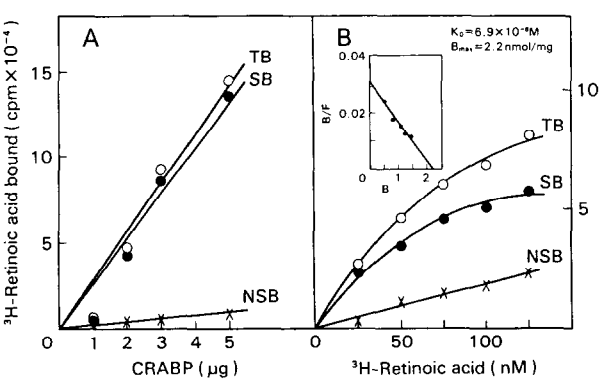

Fig.2. Specific binding of [^{3}H]retinoic acid. Various amounts of CRABP were incubated with a fixed concentration of 50 nM [^{3}H]retinoic acid (A) and 2 μg CRABP was incubated with various concentrations of [^{3}H]retinoic acid (B). Total bound [^{3}H]retinoic acid (TB,○), specifically bound [^{3}H]retinoic acid (SB,●) and nonspecifically bound [^{3}H]retinoic acid (NSB,X) were measured as described in section 2. The inset in B shows a Scatchard plot of the data [19], where B denotes the amount (nmol/mg protein) of specifically bound [^{3}H]retinoic acid and F the concentration (nM) of free [^{3}H]retinoic acid. The dissociation constant (K_d) of [^{3}H]retinoic acid from CRABP was estimated to be 6.9×10^{-8} M, and the maximum binding capacity (B_{max}) of [^{3}H]retinoic acid to be 2.2 nmol/mg CRABP. Points are means for duplicate determinations.

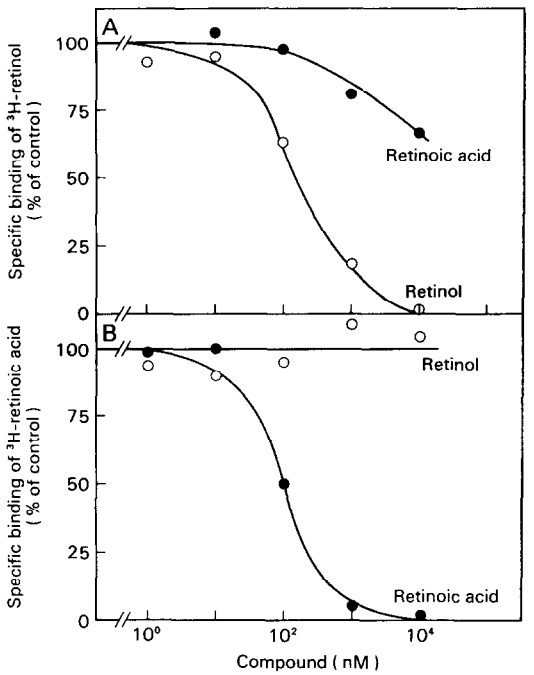

Fig.3. Inhibitions of specific binding of [^{3}H]retinol to CRBP and of [^{3}H]retinoic acid to CRABP by unlabeled retinol and retinoic acid, respectively. A mixture of 1 μg CRBP and 50 nM [^{3}H]retinol was incubated with various concentrations of unlabeled retinol (○) or unlabeled retinoic acid (●) (A). A mixture of 2 μg CRABP and 50 nM [^{3}H]retinoic acid was incubated with various concentrations of unlabeled retinol (○) or unlabeled retinoic acid (●) (B). The values, representing percent inhibitions of specific binding of tritiated ligands, are means for duplicate determinations.

CRBP, indicating the necessity for reconsidering the mode of binding of retinoids to CRBP. On the other hand, the specific binding of [^{3}H]retinoic acid to CRABP was inhibited completely by unlabeled retinoic acid, and a competition curve was obtained (fig.3B), from which the concentration of retinoic acid for 50% inhibition was shown to be about 100 nM. Nonradioactive retinol was not inhibitory over the range of concentrations examined (up to 10 μM) (fig.3B). Binding inhibition can be used to estimate the binding affinities of synthetic retinoids to cellular retinoid-binding proteins by comparison of values such as the concentrations for 50% inhibition.

The main problem in assay of binding of lipophilic ligands such as retinoids is how to separate bound from free ligands. Previously, this separation was done in an aqueous solvent. However,

rinsing with aqueous buffer resulted in a large amount of nonspecific binding of ^{3}H-ligands to the filter (not shown). To overcome this problem, in the present work we used the cold acetone filtration method. Acetone is a denaturing agent, but irreversible changes of proteins are slight when the temperature is maintained at $-78°C$, as Ashendel and Boutwell [12] reported previously. Filtration through a filter seems the most rapid method.

This is the first report of direct measurement of the specific binding of retinoids to purified CRBP and CRABP. The cold acetone filtration method is quick and practical and gives reproducible results. This method should be useful in studies on the biological function of cellular retinoid-binding proteins.

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REFERENCES

[1] Chytil, F. and Ong, D.E. (1984) in: The Retinoids (Sporn, M.B. et al. eds) pp. 89–123, Academic Press, Orlando, FL.
[2] Sani, B.P. and Hill, D.L. (1974) Biochem. Biophys. Res. Commun. 61, 1276–1282.
[3] Bashor, M.M., Toft, D.O. and Chytil, F. (1973) Proc. Natl. Acad. Sci. USA 70, 3483–3487.
[4] Ong, D.E. and Chytil, F. (1975) Nature 225, 74–75.
[5] Chytil, F. and Ong, D.E. (1976) Nature 260, 49–51.
[6] Sani, B.P. and Hill, D.L. (1976) Cancer Res. 36, 409–413.
[7] Muto, Y., Moriwaki, H. and Omori, M. (1981) Gann 72, 974–977.
[8] Küng, W.M., Geyer, E., Eppenberger, U. and Huber, P.R. (1980) Cancer Res. 40, 4265–4269.
[9] Trown, P.W., Palleloni, A.V., Bohoslawec, O., Richelo, B.N., Halpern, J.M., Gizzi, N., Gerger, R., Lewinski, C., Machilin, L.J., Jetten, A. and Jetten, M.E.R. (1980) Cancer Res. 40, 212–220.
[10] Rainier, S., Herrera, J.M. and McCormick, A.M. (1983) Arch. Biochem. Biophys. 225, 818–825.
[11] Bonelli, F.C. and De Luca, L.M. (1985) Anal. Biochem. 147, 251–257.
[12] Ashendel, C.L. and Boutwell, R.K. (1981) Biochem. Biophys. Res. Commun. 99, 543–549.
[13] Ninomiya, M., Fujiki, H., Paik, N.S., Horiuchi, T. and Boutwell, R.K. (1986) Biochem. Biophys. Res. Commun. 138, 330–334.
[14] Laemmli, U.K. (1979) Nature 227, 680–685.
[15] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[16] Ong, D.E. and Chytil, F. (1978) J. Biol. Chem. 253, 828–832.
[17] Ong, D.E. and Chytil, F. (1978) J. Biol. Chem. 253, 4551–4554.
[18] Lotan, R., Neumann, G. and Lotan, D. (1980) Cancer Res. 40, 1097–1102.
[19] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.