Jpn. J. Cancer Res. (Gann), 79, 473–483; April, 1988

Specific Uptake of Retinoids into Human Promyelocytic Leukemia Cells HL-60 by Retinoid-specific Binding Protein: Possibly the True Retinoid Receptor

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The uptake of all-*trans*-retinoic acid (RA) and two new retinoids [4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)benzoic acid (Am80) and (*E*)-4-[3-(3,5-*di-tert*-butylphenyl)-3-oxo-1-propenyl]benzoic acid (Ch55)] by HL-60 human promyelocytic leukemia cells was investigated. For the investigation, [^{3}H]RA and [^{3}H]Am80 with high specific radioactivities (more than 50 Ci/mmol) were used. [^{3}H]Am80 was prepared by hydrogenolysis of the corresponding chlorinated derivative of Am80 with tritium gas. The retinoids RA, Am80 and Ch55 were efficiently taken up by HL-60 cells, and induced differentiation of the cells into mature granulocytes. The specific bindings (uptake) of RA, Am80 and Ch55 (the bindings inhibited competitively by the other two retinoids) by HL-60 cells were due to a newly detected binding protein. The protein that bound specifically to RA appeared identical to that which bound specifically to Am80 by high-performance liquid chromatography (HPLC), and was named retinoid-specific binding protein (RSBP). One HL-60 cell was found to contain about 1500 molecules of RSBP distributed between the nuclear fraction and cytosolic fraction in proportions of about 4:1. The bindings of the three retinoids (RA, Am80 and Ch55) to RSBP (i.e., formation of retinoid-RSBP complexes) greatly enhanced the affinity of RSBP for the nuclei. The apparent molecular weight of RSBP was estimated to be 95,000 daltons by size exclusion HPLC. The association constants (*Ka*) of RSBP were calculated to be $2.4 \times 10^{10} M^{-1}$ for RA and $4.4 \times 10^{10} M^{-1}$ for Am80 from Scatchard plots. The bindings of RA, Am80 and Ch55 to RSBP were mutually competitive, indicating that the binding sites for RA, Am80 and Ch55 were identical. The very high affinities of these retinoids for RSBP (*Ka*'s of the order of $10^{10} M^{-1}$) correspond to the effective concentrations of these retinoids in HL-60 cell culture medium for induction of differentiation of the cells. The mutually competitive bindings of these retinoids strongly support the idea that RSBP is the true receptor of retinoids.

Key words: Retinoids — HL-60 — Retinobenzoic acids — Retinoid-specific binding protein — Receptor

The fundamental role of retinoids in the control of cell differentiation and cell proliferation is of interest both scientifically and clinically (e.g. in cancer prevention, cancer chemotherapy and treatment of proliferative dermatological diseases).[1–3] A retinoid is defined as a substance that can elicit specific biological responses by binding to and activating a specific receptor or set of receptors.[1, 2] The classical, potent ligand for the receptor is all-*trans*-retinoic acid (RA). The most basic and important role of retinoids is as "vitamins" for maintenance of normal growth of mammals. The specific biological responses induced by retinoids include induction of differentiation of mouse embryonal carcinoma F9 cells and human promyelocytic leukemia cells HL-60, suppression of c-*myc* expression, enhancement of the expression of the genes for epidermal growth factor receptor (EGFR) and transglutaminase, suppression of ornithine decarboxylase (ODC) activity induced by tumor promoters and of keratinization of epithelial cells, and suppression of expression of the gene for collagenase.[1–4]

Recently, we reported the synthesis of a new type of inducers of HL-60 cell differentiation, retinobenzoic acids.[5–7] These retinobenzoic acids possess retinoidal activities and so are classified as retinoids.[2–14] These retinobenzoic acids were found to be able to support growth of vitamin A-deficient rats (M. B. Sporn *et al.*, unpublished results), to induce cell differentiation of F9[12] and HL-60

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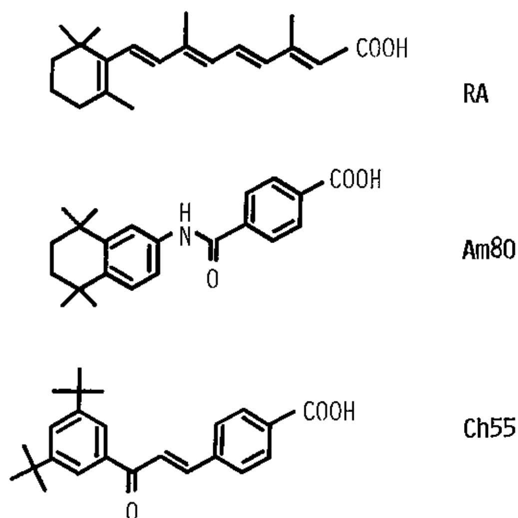

Fig. 1. Structures of RA, Am80 and Ch55.

cells,[5-11] to suppress c-*myc* expression in HL-60 cells[13] and ODC activity induced by phorbols[11] and teleocidin,[14] and to enhance the binding of epidermal growth factor (EGF) to EGFR.[2] Of the retinobenzoic acids synthesized, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)benzoic acid (Am80) and (*E*)-4-[3-(3,5-*di-tert*-butylphenyl)-3-oxo-1-propenyl]benzoic acid (Ch55) are structurally typical (Fig. 1) and possess quite high retinoidal activities.[2,5,6,11-14] As mentioned above, these retinobenzoic acids are considered to exert retinoidal activities by binding to a specific receptor. The structure-activity relationship of these retinobenzoic acids[8,9] and RA[10] strongly suggests the presence of a specific receptor for these retinoids.[4,11] One possible retinoid receptor is cellular retinoic acid binding protein (CRABP).[15] CRABP's have been found in many tissues and in cultured cells,[15-18] and although the CRABP's reported differ slightly depending on their source, they have been defined as a protein with a molecular weight of 13,000–16,000 daltons that shows specific affinity toward RA. Values for the association constant (*K*a) of CRABP with RA of $10^6-10^8 M^{-1}$ have been reported.[15,16,19] Though extensive studies have been performed on CRABP's, none of the proteins reported is likely to be the true retinoid receptor judging from the following findings.[1,3,15] (1) The retinoids Am80 and Ch55, which have stronger retinoidal activities than RA, bind to CRABP less efficiently than RA[11,12,20] (Y. Muto *et al.*, unpublished results); the affinities of Am80 and Ch55 for CRABP were estimated to be 1/80 and 1/530 of that of RA, respectively.[12] (2) In many biological assay systems, poor correlations have been found between the potencies of the biological activities of retinoids and their abilities to bind to CRABP.[11] (3) There is no correlation between the responses of cells to retinoids and the amounts (or presence) of CRABP in the cell.[1,15] In particular, no CRABP could be detected in HL-60 cells, which are very responsive to retinoids.[12,15,21,22]

The true retinoid receptor should be present in all cells or tissues that respond to retinoids, and its association constants (*K*a) with the retinoids should be consistent with their respective effective concentrations. These considerations prompted us to try to find a binding protein in HL-60 cells that had high affinities toward Am80 and Ch55 as well as RA, with *K*a values of about 10^9–$10^{11} M^{-1}$, because in typical experiments on differentiation of cultured HL-60 cells, the 50% effective dose (ED_{50}) values were $2.4\times10^{-9}M$ for RA, $7.9\times10^{-10}M$ for Am80, and $2.1\times10^{-10}M$ for Ch55.[5,6,23]

Here, we report the existence and characterization of a retinoid-specific binding protein (RSBP) with these *K*a values in HL-60 cells.

Materials and Methods

Materials [^{3}H]All-*trans*-retinoic acid ([^{3}H]RA, 52.5 Ci/mmol) was purchased from NEN Research Products. [^{3}H]4-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)-benzoic acid ([^{3}H]Am80, 65 Ci/mmol) was prepared by reduction of 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)-2,3,5,6-tetrachlorobenzoic acid (tetrachloro-Am80) with tritium gas by Amersham. Tetrachloro-Am80 was prepared from 5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylamine and terephthalic acid essentially by the method described previously.[5,23] Ch55 was prepared as described previously.[6] Scintillation cocktails (Atomlight, Aquasol-2 and Biofluor) and solubilizer (Protosol) were purchased from NEN Research Products. Ultra-filtration membranes with cut-off molecular weights of

10,000 and 50,000 daltons were purchased from Biofield Co. (Tokyo). Cellulose nitrate membranes (0.45 μm) were from Toyo Roshi Co. (Tokyo). Protease inhibitors were from Sigma Chemical Co. Coomassie blue G-250 protein assay reagent was from Pierce Chemical Co. HL-60 cells were kindly supplied by Prof. F. Takaku (Faculty of Medicine, University of Tokyo).

Cell Culture HL-60 cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) in the presence of penicillin and streptomycin at 37° in a humidified atmosphere of 5% CO_2 in air. For experiments on retinoid uptake, the cells (3×10^5 cells/ml) were incubated under the conditions described above in the presence of 0.06n*M* [^{3}H]RA or [^{3}H]Am80 in the presence or absence of 2μ*M* RA, Am80 or Ch55 for 10 hr.

Nuclear Fraction Cultured cells (ca. 10^6 cells/ml) were collected by centrifugation (1000 rpm, 5 min). The cell pellet was washed three times by resuspension in cold phosphate-buffered saline (PBS: 136.9m*M* NaCl, 2.68m*M* KCl, 8.10m*M* Na_2HPO_4, 1.47m*M* KH_2PO_4, 0.9m*M* $CaCl_2$, 0.49 m*M* $MgCl_2$). The cell pellet obtained by centrifugation was resuspended in 5% PBS in distilled water (DW) at a concentration of 3×10^5 cells/ml and left to stand at room temperature for 5 min. Then it was sonicated for 15 sec and the nuclear fraction was collected by centrifugation (600*g* for 5 min). The nuclear pellet was washed three times with cold PBS by resuspension.

Cytosol Fraction (A) The cultured cells were treated with 5% PBS in DW as described above and the supernatant obtained by centrifugation at 600*g* for 5 min was recentrifuged at 108,000*g* for 1 hr. The supernatant was used as the cytosol fraction. (B) The cultured cells were collected by centrifugation at 1000 rpm for 5 min and washed three times with cold PBS by resuspension. The cell pellet obtained by centrifugation was resuspended at a concentration of about 1×10^8 cells/ml in PBS containing a protease inhibitor cocktail[24] (PIC: 1 m*M* phenylmethylmethanesulfonyl fluoride, 1 μg/ml pepstatin, 0.1 mg/ml bacitracin, 0.1 m*M* leupeptin, 0.1 mg/ml aprotinin, 1.5m*M* EDTA). The suspension was homogenized by 9 strokes of a Teflon-glass Potter-type homogenizer at 4°. The homogenate was centrifuged at 108,000*g* for 1 hr, and the supernatant was used as the cytosol fraction.

The amount of protein in the cytosol fraction was determined with Coomassie blue G-250 as described by Bradford[25] with bovine serum albumin (BSA) as a standard. Usually, the cytosol fraction prepared from 1×10^8 cells contained 3 mg of protein.

Binding Experiments The nuclear fraction (3×10^7 nuclei/ml PBS) or cytosol fraction (1 mg protein/ml PBS) was incubated with 1.6n*M* [^{3}H]RA or [^{3}H]Am80 in the presence or absence of 2μ*M* RA, Am80 or Ch55 at 4° for 5–10 hr. The nuclear fraction was then centrifuged at 500*g* for 5 min, and the nuclear pellet was washed three times with cold PBS. The pellet was then treated with Protosol (NEN) and its radioactivity was measured in Atomlight or Aquasol-2. The amount of bound [^{3}H]retinoids in the cytosol fraction was determined by filter binding assay essentially as described by Yarus and Berg.[26] Briefly, protein in the incubation mixture was adsorbed by suction onto a nitrocellulose membrane that had been soaked in PBS. The membrane was washed three times with PBS and then with 20% ethanol in distilled water, and dried, and its radioactivity was measured in Atomlight or Aquasol-2. The specific binding was estimated by subtracting the radioactivity of samples incubated in the presence of excess cold retinoid (RA, Am80 or Ch55) from those of the corresponding samples incubated with tritium-labeled retinoids alone ([^{3}H]RA or [^{3}H]-Am80).

High-performance Liquid Chromatography (HPLC) HPLC columns were purchased from Pharmacia. Details of analytical conditions are described in figure legends. The samples injected were soluble proteins that had been incubated with [^{3}H]RA or [^{3}H]Am80 in the presence or absence of excess RA, Am80 or Ch55 as described above. The incubation mixture was concentrated by ultrafiltration (cut-off molecular weight, 10,000 or 50,000 daltons) and filtered through a 0.22 μm membrane filter. The radioactivity of the eluate was measured directly in Atomlight or Aquasol-2, or was measured by the filter binding assay described above.

RESULTS

Uptake of Retinoids by HL-60 Cells We recently reported that the retinobenzoic acids Am80 and Ch55 have the ability to induce differentiation of HL-60 cells into mature granulocytes[5,6] and to suppress c-*myc* expression in HL-60 cells.[4,13] These retinoidal activities of Am80 and Ch55 are quite strong; the relative potencies of these compounds to induce HL-60 cell differentiation are, respectively, 3.6 and 6.3 times that of RA.[5,6,23] To determine the molecular mechanisms of the retinoidal actions of these retinoids (RA, Am80 and Ch55), we first studied the uptakes of these retinoids by HL-60 cells.

Whole HL-60 cells were incubated in the presence of 0.06n*M* [^{3}H]RA or [^{3}H]Am80 at 37° for 10 hr and then separated into nuclear

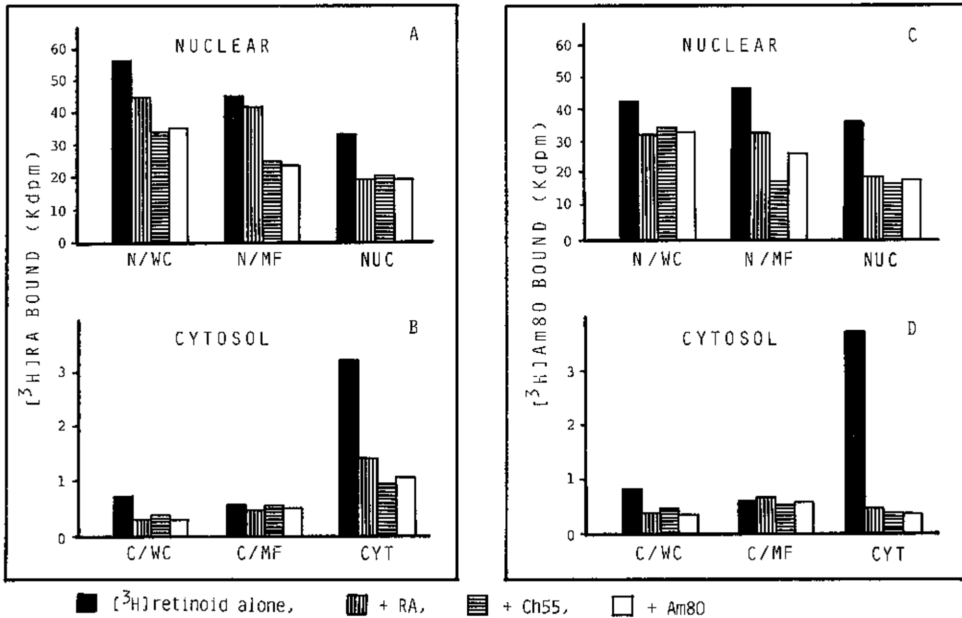

Fig. 2. Uptakes of [^{3}H]RA and [^{3}H]Am80 by HL-60 cells. Vertical scale: Bound radioactivity (kdpm) of [^{3}H]RA (A and B) and [^{3}H]Am80 (C and D) in the nuclear fraction (A and C) and the cytosol fraction (B and D) from 1×10^8 HL-60 cells. N/WC (Nuclear fraction from whole cells): HL-60 cells were incubated with [^{3}H]retinoids (0.06n*M*) in the presence or absence of 2μM RA, Am80 or Ch55 at 37° for 10 hr. Then they were fractionated into nuclear and cytosolic fractions by treatment with 5% PBS in distilled water as described in "Materials and Methods." The radioactivity bound to the nuclear fraction is shown in A and C. C/WC (Cytosol fraction from whole cells): HL-60 cells were treated as described for N/WC, and the radioactivity bound to the cytosol fraction is shown in B and D. N/MF (Nuclear fraction from the mixed fraction): The nuclear fraction and cytosol fraction were mixed and incubated with [^{3}H]retinoids in the presence or absence of excess RA, Am80 or Ch55, as described in "Materials and Methods." The incubation mixture was then refractionated into nuclear and cytosol fractions. The radioactivity bound to the nuclear fraction is shown in A and C. C/MF (Cytosol fraction from the mixed fraction): Samples were treated as for N/MF. The radioactivity bound to the cytosol fraction is indicated in B and D. NUC: The nuclear fraction was incubated with [^{3}H]retinoids in the presence or absence of excess RA, Am80 or Ch55 as described in "Materials and Methods," and its radioactivity is shown in A and C. CYT: The cytosol fraction was incubated with [^{3}H]retinoids in the presence or absence of excess RA, Am80 or Ch55 as described in "Materials and Methods," and its radioactivity is shown in B and D.

and cytosol fractions. The amounts of [^{3}H]-retinoids bound to macromolecules in these fractions were measured (Fig. 2, N/WC and C/WC). Similar results were obtained with [^{3}H]RA (Fig. 2, A and B) and [^{3}H]Am80 (Fig. 2, C and D). Under the incubation conditions used, about 10% of the added [^{3}H]-retinoids was taken up by HL-60 cells, and the concentration of [^{3}H]retinoids in the cells became 200 times that in the medium. The ratio of the uptakes of [^{3}H]retinoids by the nuclear and cytosolic fractions was about 60:1. The amounts of specific binding were estimated by displacing radioactivity by simultaneous addition of excess (2μM) cold retinoids (RA, Am80 or Ch55). As shown in Fig. 2 (N/WC and C/WC), approximately 60–80% of the binding was non-specific. Scatchard analysis indicated that HL-60 cells have low-capacity, high-affinity binding sites (*Ka*, 10^9–$10^{10}M^{-1}$; approximately 1500 binding sites per cell) and high-capacity, low-

affinity binding sites (*K*a, 10^6–$10^7 M^{-1}$; more than 10^8 binding sites per cell) (data not shown); the former sites were specific and the latter, non-specific. The specifically bound [^{3}H]RA and [^{3}H]Am80 could be efficiently displaced by simultaneous addition of unlabeled preparations of any other retinoid, RA, Am80 or Ch55. This suggested that the specific bindings of RA, Am80 and Ch55 were mutually competitive. Incubation in the presence of higher concentrations of [^{3}H]-retinoids (2n*M* or more) resulted in undetectable specific binding (data not shown), because the several orders higher amount of non-specific binding than specific binding precluded detection of the latter.

The results of binding experiments using fractionated cells (nuclear fraction and cytosol fraction) are shown in Fig. 2 (NUC and CYT). In these experiments, the nuclear and cytosol fractions prepared from cultured HL-60 cells were incubated separately with [^{3}H]retinoids. The ratio of the amounts of specific binding in the nuclear and cytosol fractions was about 4:1. This indicated a somewhat higher proportion of specific binding in the cytosol fraction than that determined in the experiment described above using whole cells (WC), although the total amount of specific binding was approximately the same in the two experiments. This suggests that the macromolecule bound with retinoids has higher affinity than the free macromolecule for nuclei. To confirm this increase in affinity of the macromolecule when it is bound to retinoids, we combined the nuclear and cytosol fractions and incubated the mixture with [^{3}H]retinoids (Fig. 2, N/MF and C/MF). The mixture was then refractionated and radioactivities were measured. As expected, scarcely any specific binding was detected in the cytosol fraction (Fig. 2, C/MF), though it was detected in the nuclear fraction (Fig. 2, N/MF). These results suggest that this macromolecule (which combines specifically with retinoids) binds to nuclei ligand-dependently.

The specific binding activity was lost when the fractions were treated with trypsin or heat (60°, 10 min) (data not shown). This suggests that this macromolecule that binds specifically to retinoids is proteinous. This protein did not pass through an ultra-filtration membrane with a cut-off molecular weight of 50,000 daltons, which suggests that its molecular weight is more than 50,000 daltons. In the fraction of molecular weights less than 50,000 daltons, no specific binding was detectable. The result supports the absence of CRABP, whose molecular weight is about 13,000–16,000 daltons, in HL-60 cells, as reported by other authors.[12, 15, 21, 22]

The specific binding activity associated with the nuclear fraction could be solubilized by treatment with high-salt solution (2*M* NaCl), a detergent (Triton X-100, 1%), sonication, or DNase I, though the recovery of the activity was low (data not shown).

The results of these binding experiments suggest that there are about 1500 molecules of retinoid-specific binding protein (RSBP) per HL-60 cell, and that they are distributed between the nuclear and cytosol fractions in a ratio of about 4:1.

HPLC Analysis of RSBP RSBP in the cytosol fraction prepared from cultured HL-60 cells was analyzed with HPLC. The cytosol fraction was incubated with [^{3}H]RA or [^{3}H]-Am80 in the presence or absence of excess cold retinoid (RA, Am80 or Ch55). The incubation mixture was then concentrated by ultrafiltration (cut-off molecular weight, 50,000 daltons) and separated by size exclusion HPLC (Fig. 3) and anion exchange HPLC (Fig. 4).

The chromatogram of a sample incubated with [^{3}H]RA (Fig. 3, B and Fig. 4, B) indicated the presence of several peaks with radioactivity which is displaced by the addition of excess unlabeled RA; these peaks are those of retinoic acid-specific binding proteins. However, the radioactivity of only one of these peaks (retention time (T_R,) 27–28 min in Fig. 3, B and 24 min in Fig. 4, B) was displaced by addition of excess cold Am80 or Ch55. Therefore, there is only one major peak of retinoid-specific binding protein in the cytosol fraction of cultured HL-60 cells, though there are several retinoic acid-specific binding proteins.

The chromatogram of a sample incubated with [^{3}H]Am80 (Fig. 3, C and Fig. 4, C) was simpler: only one major peak with radioactivity was detected. The radioactivity of the peak was displaced by addition of excess unlabeled RA, Am80 or Ch55. The T_R values of the

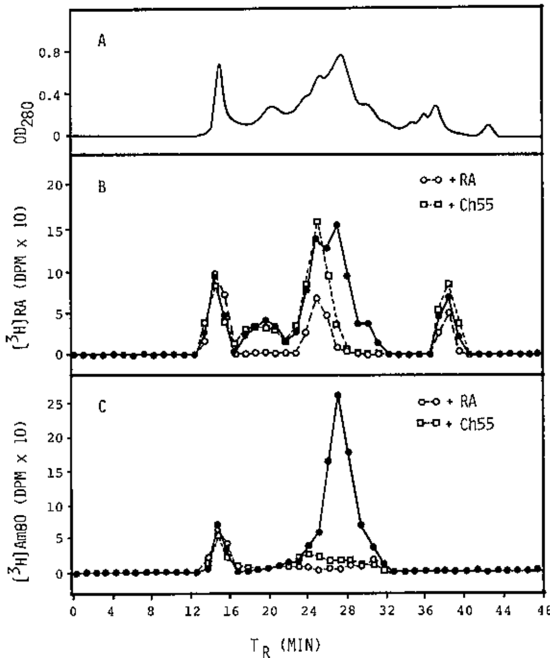

Fig. 3. Size exclusion HPLC of cytosol incubated with [^{3}H]retinoids. Column: Superose 12 HR 10/30 (Pharmacia). Eluent: 50mM Phosphate (pH 7.2)–0.15M NaCl. Flow rate: 0.5 ml/min. A: Monitored by measuring the absorbance at 280 nm. All injected samples gave similar chromatographic patterns. B and C: The cytosol fraction prepared from HL-60 cells was incubated with [^{3}H]RA (B) or [^{3}H]Am80 (C) in the absence (●) or presence of excess RA (○) or Ch55 (□) as described in "Materials and Methods." The radioactivity of each fraction (one fraction = 0.5 ml) was measured in Aquasol-2. The chromatogram of the sample incubated with [^{3}H]retinoids in the presence of excess Am80 was similar to that of the sample incubated with excess Ch55 (□).

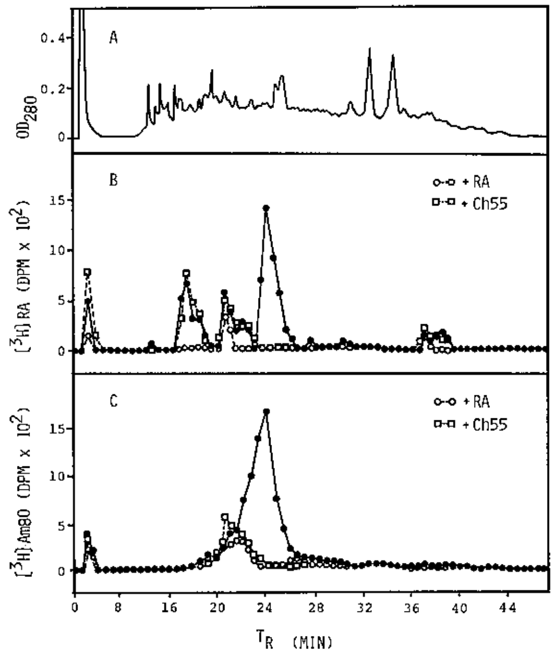

Fig. 4. Anion exchange HPLC of cytosol incubated with [^{3}H]retinoids. Column: Mono Q HR 5/5 (Pharmacia). Eluent: A linear gradient of 0–0.5 M NaCl (10mM NaCl increase/min) in 20mM Tris-HCl (pH 8.0). Flow rate: 1.0 ml/min. A: The same as described for Fig. 3 (A). B and C: The same as described for Fig. 3 (B and C), except that one fraction was 1.0 ml and the radioactivity was measured in Atomlight.

peak were identical in the two HPLC analyses (Fig. 3, B and C, and Fig. 4, B and C) with those of the peak of specific binding in the sample incubated with [^{3}H]RA. This identity of the peak strongly suggests that the protein detected in samples incubated with [^{3}H]RA and with [^{3}H]Am80 is the retinoid-specific binding protein (RSBP).

Samples with and without incubation with [^{3}H]retinoids gave identical chromatograms, monitored as absorbance at 280 nm. Specific activities of binding retinoids was detected in the fraction with a T_R value of 27–28 min on size exclusion HPLC and a T_R of 24 min on anion exchange HPLC (these T_R's were identical with those of the radioactivity of the retinoid-RSBP complex). These results suggest that free RSBP and the retinoid-RSBP complex behave similarly on HPLC analysis.

On both HPLC's, the recovery of retinoid-specific binding activity was more than 70%. The RSBP was concentrated 20- to 50-fold by anion exchange HPLC (Fig. 4). The chromatogram of the fraction collected by anion exchange HPLC (Fig. 4, T_R= 23–26 min) is shown in Fig. 5. The chromatograms on size exclusion HPLC (Fig. 5, B and C) and hydrophobic interaction HPLC (Fig. 5, E and F) show essentially a single radioactive peak. Successive separation of RSBP by anion exchange HPLC (Fig. 4) and hydrophobic interaction HPLC (Fig. 5, D–F) and size exclu-

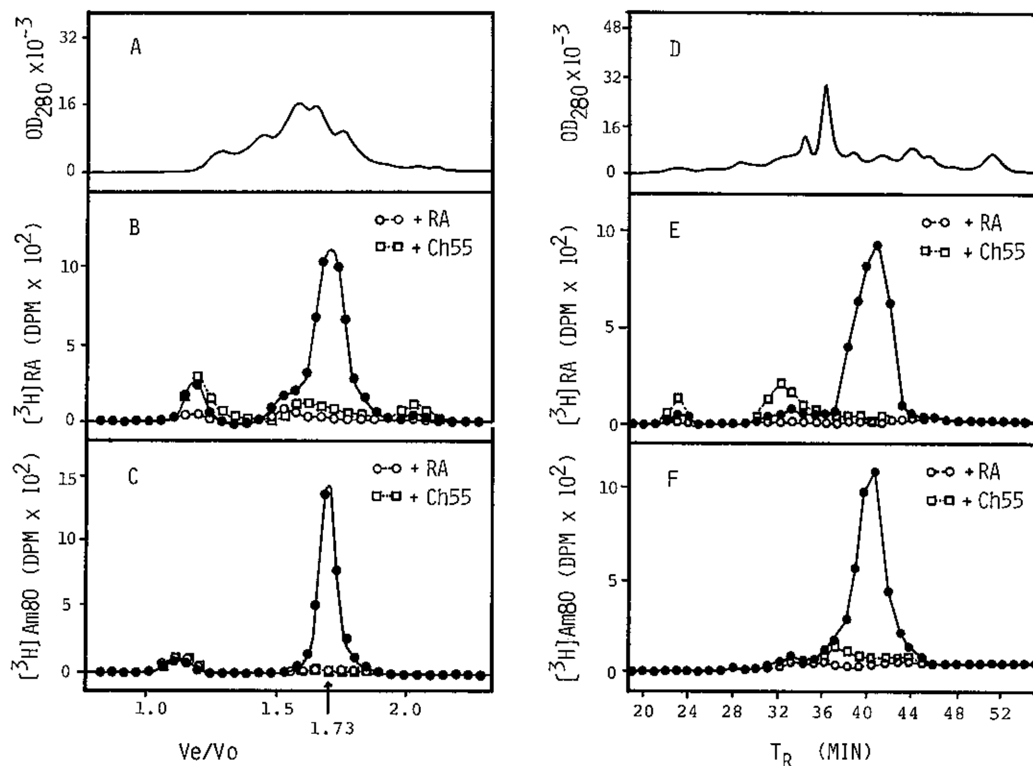

Fig. 5. Chromatogram of [^{3}H]retinoid-RSBP complex. Sample: Fractions containing [^{3}H]RA-RSBP complex (B and E) or [^{3}H]Am80-RSBP complex (C and F) separated by anion exchange HPLC (Fig. 4, T_R of 23–26 min) and concentrated by ultrafiltration (cut-off molecular weight, 50,000 daltons). Column: Superose 12 HR 10/30 (Pharmacia) for A–C and Phenyl-Superose HR 5/5 for D–F. Flow rate: 0.5 ml/min. Eluent: The same as for Fig. 3 (A–C) or a linear gradient of 1.7–0*M* $(NH_4)_2SO_4$ (34m*M* $(NH_4)_2SO_4$ decrease/min) in 50m*M* phosphate (pH 7.0) (D–F). A and D: Monitored by measuring the absorbance at 280 nm. B, C, E and F: The same as for Fig. 3 except that the radioactivity of each fraction was measured in Atomlight. The void volume of the size exclusion HPLC column (A–C) was determined by injection of blue dextran (2,000,000 daltons).

sion HPLC resulted in more than 500-fold concentration of RSBP with a recovery of retinoid-specific binding activity of more than 30%.

Estimation of Molecular Weight and the Association Constant of RSBP For characterization of RSBP, we examined its molecular weight and association constants (*K*a's) with retinoids. The apparent molecular weight of RSBP was estimated by size exclusion HPLC of partially purified RSBP incubated with [^{3}H]RA or [^{3}H]Am80 (Fig. 5, A–C and Fig. 6). A calibration curve of molecular weight versus Ve/Vo was obtained with molecular weight size markers, and from the Ve/Vo of 1.73 for the [^{3}H]retinoid-RSBP complex, its molecular weight was estimated to be 95,000 daltons.

The association constants (*K*a's) of RSBP with RA and Am80 were estimated by Scatchard analysis (Fig. 7). The partially purified RSBP was incubated with various concentrations of [^{3}H]RA or [^{3}H]Am80 at 4° for 2 hr, and then their binding was measured by filter binding assay.[26,27] Under the experimental conditions used, good linearity was obtained by the least-squares method ($r = -0.997$ for [^{3}H]Am80 and $r = -0.990$ for

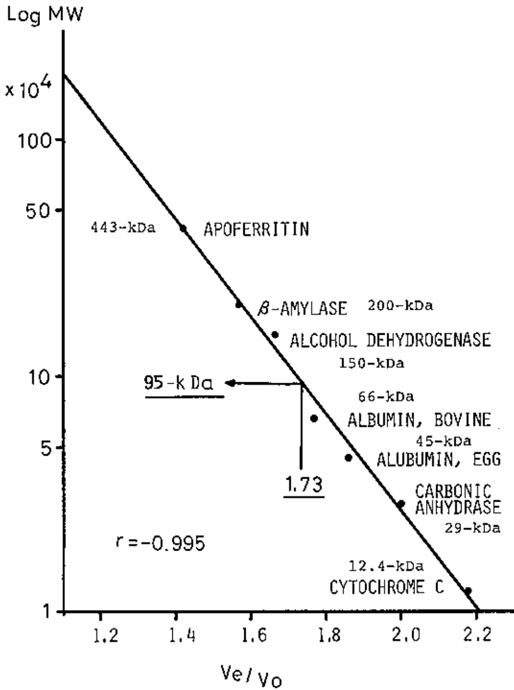

Fig. 6. Estimation of apparent molecular weight of RSBP. Ve/Vo was determined by size exclusion HPLC under the conditions described in the legends to Figs. 3 and 5 (A–C). The Ve/Vo of RSBP was 1.73. The calibration curve was obtained by the least-squares method ($r=-0.995$) by injection of the molecular weight markers indicated in the figure.

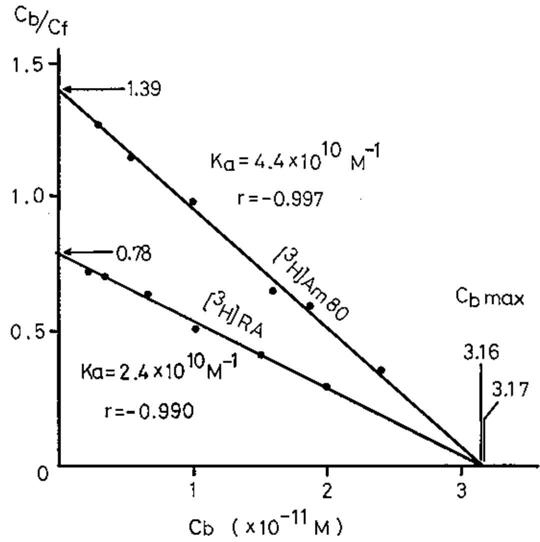

Fig. 7. Scatchard analysis of the [^{3}H]retinoid-RSBP complex. Cb: Concentration of bound [^{3}H]-retinoids determined from the radioactivity by filter binding assay as described in "Materials and Methods." Cf: Concentration of free [^{3}H]retinoids determined from the radioactivity of the filtrate of the filter binding assay. Cb max: Concentration of binding sites for [^{3}H]retinoids under the experimental conditions. *Ka*: Association constants calculated from the slope of the regression curve drawn by the least-squares method.

[^{3}H]RA, Fig. 7). The *Ka*'s were estimated as $4.4\times10^{10}M^{-1}$ for the [^{3}H]Am80-RSBP complex and $2.4\times10^{10}M^{-1}$ for the [^{3}H]RA-RSBP complex. Thus RSBP had 1.8-fold higher affinity for Am80 than for RA. The concentrations of binding sites under these experimental conditions were $3.16\times10^{-11}M$ for Am80 and $3.17\times10^{-11}M$ for RA (identical within the limits of experimental error). This coincidence of these values for the concentration of the binding sites, and the reciprocal competitive binding of RA and Am80 strongly suggest that RA and Am80 have the same binding site. The concentration of the binding sites obtained in these experiments ($3.16–3.17\times10^{-11}M$) corresponds to the amount of RSBP in the cytosol per HL-60 cell (350–370 molecules). This amount estimated by Scatchard analysis is consistent with the amount estimated in the binding experiments using fractionated cells described above (about 1500 molecules of RSBP per HL-60 cell with a ratio of distributions in the nuclear and cytosol fractions of about 4:1).

Discussion

As mentioned before, a retinoid is defined biologically as a substance that elicits specific biological responses by binding to and activating a specific receptor or set of receptors.[1,2] This definition indicates that in studies on the molecular mechanism of retinoidal action, attention should be focused on the receptor system. So far, little is known about the receptor molecules, in spite of extensive studies on cellular retinoid-binding protein (e.g., on CRABP).[15] CRABP's are unlikely to be true receptors, although they may have roles in cellular transport, storage or metabolism of retinoids.[1] Therefore, we tried to identify the

true retinoid receptor by investigating the uptake of retinoids by HL-60 cells. For this purpose, we used not only RA, but also Am80 and Ch55, which were recently synthesized as examples of a new type of inducer of differentiation of HL-60 cells,[5,6,11] and were classified as retinoids because they have the same biological activities as RA.[2,4,11-14] HL-60 cells respond actively to retinoids and in this work we demonstrated that they contain a retinoid-specific binding protein (RSBP), to which RA, Am80 and Ch55 bind specifically and competitively. The true retinoid receptor should be present in cells or tissues that respond to retinoids and should show high specific affinity for all retinoids, so the RSBP demonstrated here fulfills the criteria for the true receptor.

Specific uptake of RA by HL-60 cells has not been recognized so far, probably because the capacity of non-specific binding of RA is several orders higher.[12,15,21,22] We overcame this difficulty by using very dilute [^{3}H]-retinoids of high specific radioactivity (more than 50 Ci/mmol), so we were able to detect specific binding of retinoids in HL-60 cells. We found that there are about 300–400 molecules of RSBP in the cytosol, and about 1200 molecules of RSBP in the nucleus per HL-60 cell. The association constants (*K*a's) of RSBP with the retinoids (RA, Am80 and Ch55) were estimated to be of the order of $10^{10}M^{-1}$. The concentration of RSBP in HL-60 cells and the *K*a's of RSBP with the retinoids are consistent with the high ability of the cells to respond to retinoids and the effective concentration of retinoids to elicit responses of these cells (RA, Am80 and Ch55 induce differentiation of HL-60 cells at concentrations of 10^{-10}–$10^{-9}M$).[5,6,23] The 1.8-fold higher *K*a of RSBP for Am80 than for RA is also consistent with the 3.6-fold higher potency of the former to induce differentiation of HL-60 cells.[5,23] These results support the idea that the RSBP is the true receptor of retinoids.

RA, Am80 and Ch55 bind to the same site of RSBP. However, HL-60 cells contain a large amount of non-specific binding sites for RA. In HPLC analysis, Am80 proved far superior to RA for studies on RSBP, because its specificity of binding to RSBP is higher than that of RA, as shown in Figs. 3 and 4. Moreover, Am80 is more stable than RA both chemically (stability in acid) and physically (stability to heat and light). The tritium-labeled Am80 of high specific radioactivity used in this work should be helpful in studies on the molecular mechanism of retinoidal action.

We expect that retinoids with similar biological activities (retinoidal actions) share a common mechanism of action. At present, the most likely mechanism seems to be similar to that proposed for the action of steroids.[28-30] Chytil and Ong suggested a role for the binding proteins in the translocation of retinoids to specific sites on the chromatin resulting in specific modulation of gene transcription.[30] CRABP's are unlikely to be the true receptors, but specific interaction of a retinoid-CRABP complex with chromatin has been demonstrated.[31] The retinoid-dependent binding of RSBP to nuclei demonstrated in this paper provides support for a steroid-like mechanism of action of retinoids. The apparent molecular weight of RSBP (95,000 daltons) and the very high *K*a (of the order of $10^{10}M^{-1}$) of RSBP with retinoids are comparable with those reported for steroid receptors.[28] In order to clarify this mechanism, RSBP must be isolated. We are now trying to isolate it by affinity column chromatography with a Ch55-like ligand. Very recently, Petkovich *et al.* reported the cloning of a cDNA encoding a protein (48,000 daltons) that binds to RA with a *K*a of more than 10^8 M^{-1}.[32] The relation between this protein[32] and RSBP is of interest.

The effects of retinoids on cell growth can be partially interpreted in terms of the effects of these compounds in suppressing c-*myc* expression, enhancing the expression of the gene of epidermal growth factor receptor (EGFR) and regulating other important genes.[4] The retinoid-RSBP complex may bind specifically to the regions regulating these genes. The sites of binding of the retinoid-RSBP complex to this locus of the genes must be identified.

In summary, we have identified a retinoid-specific binding protein (RSBP) (apparent molecular weight, 95,000 daltons; *K*a for RA, $2.4\times10^{10}M^{-1}$, *K*a for Am80, $4.4\times10^{10}M^{-1}$). RA, Am80 and Ch55 bind to the same site of this RSBP in HL-60 cells in a mutually competitive manner. RSBP is distributed between

the nuclear and cytosol fractions of HL-60 cells in a ratio of about 4:1, and the affinity of RSBP for nuclei is greatly enhanced by formation of a retinoid-RSBP complex. The retinoid-RSBP complex is rather stable and could be separated by various types of HPLC. [^{3}H]Am80, synthesized for the first time, proved very useful in these experiments. [^{3}H]-Am80, Am80 and Ch55 should all be useful in investigations on retinoidal action.

ACKNOWLEDGMENTS

This work was supported by Grant-in-Aid from Special Project Research on Cancer Bio-Science by the Ministry of Education, Science and Culture, Japan.

(Received Dec. 25, 1987/Accepted Jan. 26, 1988)

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