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HIGH PRESSURE LIQUID CHROMATOGRAPHIC DETECTION OF INTRACELLULAR RETINOID BINDING PROTEINS FROM CULTURED CELL AND TUMOR CYTOSOLS

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We report the first application of high pressure liquid chromatography (HPLC) in the rapid detection of cellular retinoic acid binding protein (CRABP) and cellular retinol binding protein (CRBP). Cytosols from cultured cells (3T6 and MCF-7) or from tumors (melanoma and ovarian) were labeled with [^{3}H]retinoic acid (30 Ci/mmol) and [^{3}H]retinol (43 Ci/mmol) and analyzed via HPLC employing a 60 cm TSK 3000 sw column. In each case CRABP and CRBP were readily detectable at an elution volume of 22.5 ml, consistent with their molecular weights of 14,600. Identity of the binding protein peaks was established by saturability, specificity, and selective inhibition of binding by an organomercurial. Thus, this method, which resolves CRABP and CRBP in crude mixtures from the majority of cytosolic proteins, should be a valuable tool in the evaluation of vitamin A-binding protein interactions and their biological significance.

There are two unique cytosolic proteins of molecular weight 14,600 found in many cells that specifically bind retinoic acid and retinol (1). These proteins are cellular retinoic acid binding protein (CRABP) and cellular retinol binding protein (CRBP). CRABP and CRBP bind their respective ligands with high affinity (K_d=4-16 nM) and, in the case of CRABP, there is a strong correlation between the binding affinities of a series of retinoic acid analogues and their biological activities, *in vitro* and *in vivo* (2). Thus, currently it is thought that CRABP and CRBP may be the intracellular mediators of retinoid action that control cell growth and differentiation (3). Although the mechanism of this action is not completely understood, the binding proteins apparently deliver retinol (4) and retinoic acid (5) to the nucleus,

Abbreviations used: HPLC, high pressure liquid chromatography; CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol binding protein; ET buffer, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4; pCMBS, p-chloromercuribenzene sulfonate; DMEM, Dulbecco's minimum essential medium; PBS, phosphate buffered saline.

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where they could affect both replication and transcription. Antiproliferative effects of retinoic acid have been demonstrated in a number of cancer cell types including breast (6), melanoma (7), and leukemia (8). This growth inhibition is often linked to differentiation to a more normal phenotype. Moreover, evidence is accumulating from experiments in both animals and humans that vitamin A has potent anticancer effects (9,10). Since it is likely that CRABP and CRBP play a molecular role in the antineoplastic action of retinoids, the detection of these binding proteins in tumors and tumor cell lines is of considerable interest. CRABP and CRBP have previously been analyzed by using radiolabeled retinoid and time consuming sucrose gradient determination of the 2S macromolecules (5,6) or by polyacrylamide gel electrophoresis (11). Sani and Banerjee (12) have noted that CRABP and CRBP association with retinoids is inhibited by organomercurials. We utilized this fact along with the recently improved high pressure liquid chromatographic (HPLC) protein separation procedures to devise a rapid and unequivocal method for revealing the presence of these retinoid binding proteins in biological samples.

MATERIALS AND METHODS

Culture and Tumor Preparation. MCF-7, human breast carcinoma cells were a generous gift from Dr. Larry McReynolds (New England Biolabs, Beverly, MA). Cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10 percent newborn calf serum and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). 3T6, embryonic mouse fibroblast cells were obtained from the American Type Culture Collection. Cells were grown in DMEM supplemented with 10 percent fetal bovine serum and antibiotics. Cultures were incubated at 37°C in a 95 percent air/5 percent CO_2 humidified atmosphere. Cells were harvested at confluency by standard trypsinization procedures and washed in Dulbecco's phosphate buffered saline (PBS) to remove any serum contamination.

Tumor specimens consisted of a histopathologically confirmed human ovarian tumor obtained at surgery and a mouse melanoma tumor. The melanoma was induced with MEL-11A cells (a melanotic subclone of Cloudman S91 melanoma cells obtained from Dr. Bryan Fuller, Department of Biology, Texas Tech University, Lubbock, TX) that were cultured as described elsewhere (13), injected subcutaneously into a genetically susceptible mouse (DBA/2J), and harvested after 2 weeks as a 1.18 g tumor.

Cytosol preparation. 3T6 (45×10^6) or MCF-7 (133×10^6) cells were suspended in 1 ml of ET buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.4) and sonicated to disrupt the cell membrane. The sonicate was then centrifuged at 159,000 x g for 45 min to yield the cytosolic supernatant. Tumors (1 g) were rinsed in PBS, homogenized with a Polytron (Brinkmann Instruments) in 4 ml ET buffer and centrifuged at 12,000 x g for 5 min. The supernatant was decanted and ultracentrifuged at 159,000 x g for 45 min to obtain the cytosol.

Cytosol labeling with retinoids. [^{3}H]retinoic acid (30 Ci/mmol) was generously supplied by Hoffmann LaRoche Inc. (Nutley, NJ) and [^{3}H]retinol (43 Ci/mmol) was a gift from Amersham International (Arlington Heights, IL). [^{3}H]retinoids were stored in a solution of toluene/ethanol (1:1) and were greater than 85 percent radiochemically pure when analyzed by reverse phase HPLC (14,15). All trans-retinoic acid and all trans-retinol were purchased from Sigma Chemical Co. (St. Louis, MO). All solutions of retinoids were stored at -20°C in aluminum foil shielded vials purged with nitrogen gas to protect against photoisomerization and oxidation. In each experiment retinoids were pipetted into 16 x 100 mm borosilicate tubes under very dim light and dried with a stream of nitrogen gas. Cytosol (250 µl), diluted with either ET buffer (350 µl), or 350 µl ET buffer containing 4 mM p-chloromercuribenzene sulfonate (pCMBS), was added to each retinoid containing tube and incubation was carried out in the dark at 4°C for 24 hr.

HPLC. A Beckman HPLC-model 322 equipped with a 0.75 x 60 cm TSK 3000 sw gel permeation column was utilized. Mobile phase buffer was 20 mM K_2HPO_4, 0.2 M KCl, pH 7.0. Labeled cytosol (200 µl) was directly injected onto the column and chromatographed at a flow rate of 1 ml/min. Absorbance was monitored at 280 nm and thirty second fractions were collected for a total period of 30 min. Radioactivity in each fraction was measured with a Beckman LS233 or LS250 liquid scintillation counter employing a toluene based aqueous counting solution (efficiency = 36 percent).

RESULTS AND DISCUSSION

3T6 cytosol, previously determined (15,16) to contain CRABP (47 pmol/mg protein) and CRBP (21 pmol/mg protein), was used to test the ability of gel permeation HPLC to detect these binding proteins. [^{3}H]retinoic acid labeled cytosol (Fig. 1A-C) yielded several peaks of radioactivity, one at 22.5 min which was separated from the majority of UV absorbing substances and was judged to represent specific CRABP binding activity (Fig. 1A) because of its inhibitability by pCMBS (Fig. 1B). The selectivity of pCMBS blocking of retinoic acid binding to CRABP is illustrated by the lack of effect of the organomercurial on retinoic acid appearance in the regions of 10-12 and 23-30 min. Based upon chromatography of standard proteins (data not shown), an elution time of 22.5 min for CRABP is consistent with its molecular weight of 14,600. Furthermore, identification of CRABP was confirmed by the observation that the 22.5 min peak was completely abolished when incubation of cytosol was carried out in the presence of a 200 fold excess of nonradioactive retinoic acid, but not by the presence of a 200 fold excess of radioinert retinol (Fig. 1C). Elimination of the 22.5 min peak by excess retinoic acid demonstrates the presence of a finite number of saturable, high affinity binding sites which are specific for retinoic acid since they are not influenced by retinol.

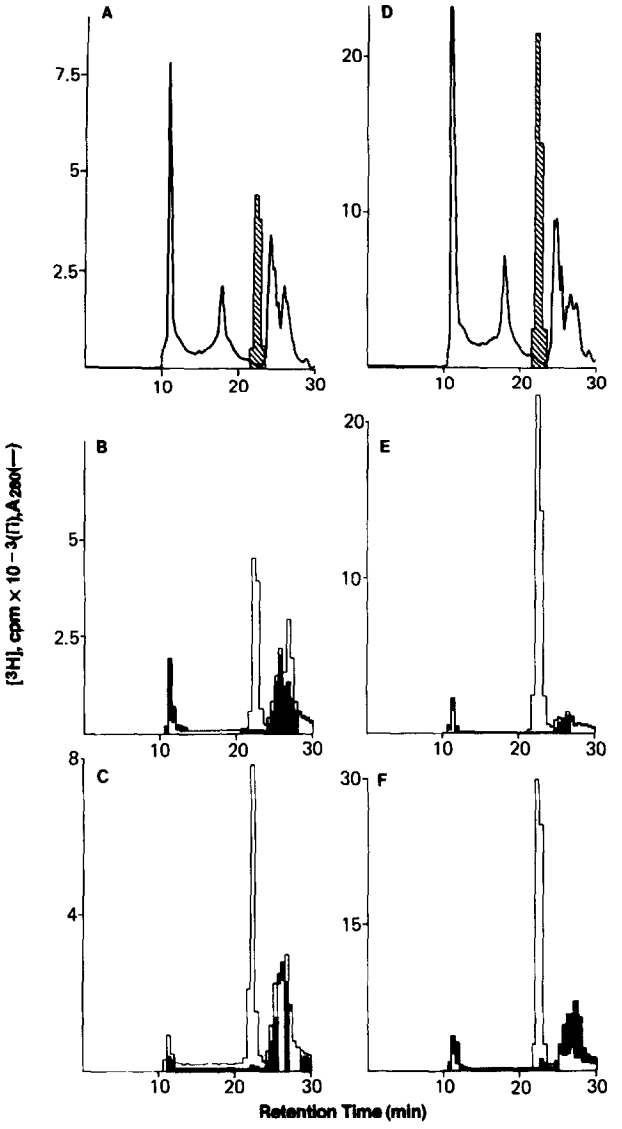

Figure 1. HPLC identification of retinoid binding proteins in 3T6 cytosol. A-C: cytosol labeled with 35 nM [^{3}H]retinoic acid. A: UV (280 nm) protein profile (—) and specific binding (▨) as determined by the difference in radioactivity in the absence and presence of pCMBS. B: Total radioactivity bound to proteins (Π) and radioactivity after addition of pCMBS (■). C: Radioactivity bound in the presence of a 200 fold excess of unlabeled retinol (Π) or unlabeled retinoic acid (■). D-F: Cytosol labeled with 50 nM [^{3}H]retinol. D and E: See A and B above. F: Radioactivity bound in the presence of a 200 fold excess of unlabeled retinoic acid (Π) or unlabeled retinol (■). Standards: Blue Dextran (11 min), BSA (17.5 min), myoglobin (22.4 min), total column volume (28 min).

Similarly, CRBP, which was formed by incubation with [^{3}H]retinol, can be resolved from the majority of 3T6 cytosolic proteins by HPLC (Fig. 1D-F). CRBP shares two properties with CRABP, namely, a retention time of 22.5 min

(MW = 14,600) and inhibitability by pCMBS (Fig. 1D-E). However, CRBP can be uniquely identified since the peak is obliterated by incubation with excess unlabeled retinol, but not with unlabeled retinoic acid (Fig. 1F).

We applied this methodology to MCF-7, a human breast cancer cell line, known to contain CRABP and CRBP (6) in lower concentration than the 3T6 fibroblast cells (our unpublished data). Fig. 2A-C depicts CRABP analysis of MCF-7 cytosol by HPLC and reveals a peak of [^{3}H]retinoic acid binding at 22.5 min with chemical and physical properties indistinguishable from those of 3T6 CRABP, which were summarized in Fig. 1A-C. In contrast, another human cancer cell line, HL-60 (promyelocytic leukemia), exhibited no detectable CRABP when analyzed identically to MCF-7. In spite of that fact that HL-60 differentiates in the presence of retinoic acid (8), the present finding is consistent with the apparent absence of CRABP in this cell line (17). Thus, in the case of HL-60, one must consider CRABP-independent mechanisms of retinoic acid action, such as involvement by vitamin A metabolites in glycosyl transferase reactions in a manner similar to dolichol phosphate (18).

Fig. 2D-F represents direct analysis of tumor tissue cytosol for CRBP. In this instance, the tissue source was an induced mouse melanoma which received no treatment to remove blood and other contaminants. CRBP is clearly detectable in the melanoma tumor and has properties similar or identical to those of CRBP from 3T6 cells (Fig. 1D-F). A human ovarian tumor containing 3 pmol CRABP/mg cytosol protein (15) was assayed in the same manner and displayed a 3500 cpm peak of specific [^{3}H]retinoic acid binding at 22.5 min retention time (data not shown). Hence, unlike other methods, this HPLC technique of direct injection of labeled cytosol is capable of detecting both CRABP and CRBP in crude tumor specimens.

Previously, HPLC has been utilized for the detection and/or purification of a number of biologically important peptides such as growth factors (19), steroid hormone receptors (20), and 1,25-dihydroxyvitamin D_3 receptors (21). We demonstrate that this method is appropriate for the detection of retinoid binding proteins in unpurified cytosolic fractions and is rendered

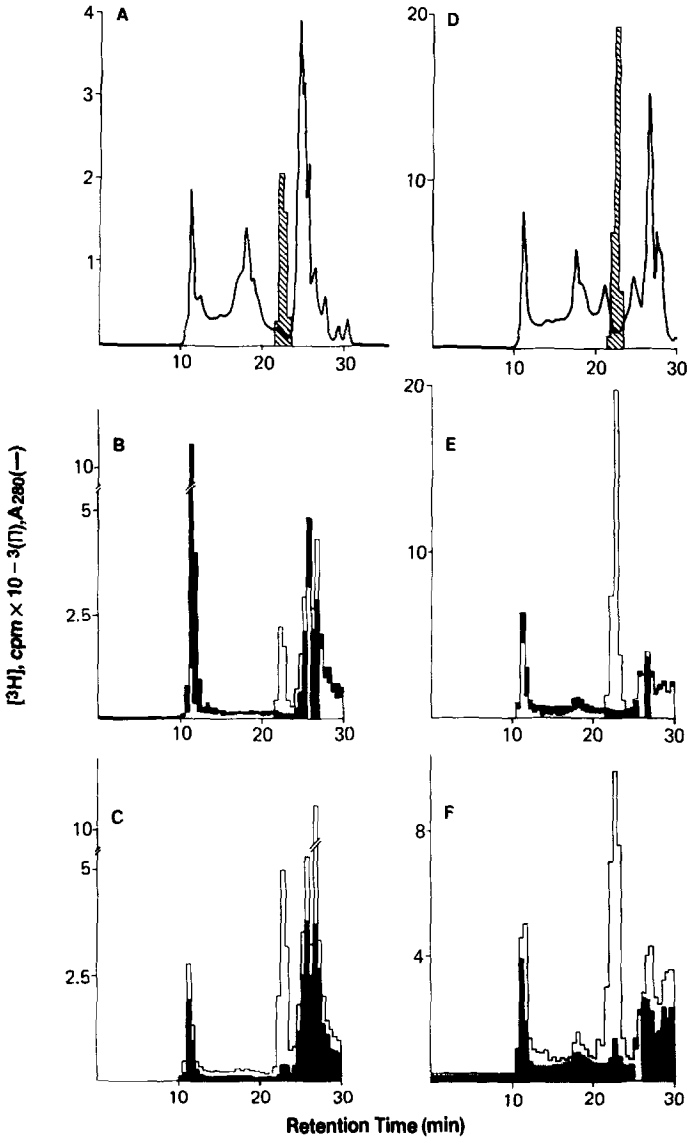

Figure 2. HPLC identification of retinoid binding proteins in MCF-7 and melanoma tumor cytosols. A-C: MCF-7 cytosol labeled with 75 nM [^{3}H]retinoic acid. A: UV (280 nm) protein profile (—) and specific binding (▨) as determined by the difference in radioactivity in the absence and presence of pCMBS. B: Total radioactivity bound to proteins (Π) and radioactivity after addition of pCMBS (■). C: Radioactivity bound in the presence of a 200 fold excess of unlabeled retinol (Π) or unlabeled retinoic acid (■). D-F: Mouse melanoma tumor cytosol labeled with 75 nM [^{3}H]retinol. D and E: See A and B above. F: Radioactivity bound in the presence of a 200 fold excess of retinoic acid (Π) or unlabeled retinol (■). See standards in Fig. 1.

sensitive by the use of high specific activity (30-43 Ci/mmol) [^{3}H]retinoids. Our results are consistent with published data derived from other methods, on both CRABP and CRBP in cultured cells and tumors. This

establishes the validity of HPLC for retinoid binding protein analysis and suggests that it will be a powerful tool in unraveling the molecular biology of vitamin A action and evaluating its potential role as an anticancer agent.

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REFERENCES

1. Chytil, F., and Ong, D.E. (1979) Fed. Proc. 38, 2510-2514.
2. Trown, P.W., Palleroni, A.V., Bohoslawec, O., Richelo, B.N., Halpern, J.M., Gizzi, N., Geiger, R., Lewinski, C., Machlin, L.J., Jetten, A., and Jetten. M.E.R. (1980) Cancer Res. 40, 212-220.
3. Lotan, R. (1980) Biochim. Biophys. Acta 605, 33-91.
4. Liau, G., Ong, D.E., and Chytil, F. (1981) J. Cell Biol. 91, 63-68.
5. Mehta, R.G., Cerny, W.L., and Moon, R.C. (1982) Biochem. J. 208, 731-736.
6. Lacroix, A., and Lippman, M.E. (1980) J. Clin. Invest. 65, 586-591.
7. Meyskens, F.L. Jr., and Fuller, B.B. (1980) Cancer Res. 40, 2194-2196.
8. Breitman, T.R., Selonick. S.E., and Collins, S.J. (1980) Proc. Natl. Acad. Sci. USA 77, 2936-2940.
9. Sporn, M.B., and Newton, D.L. (1979) Fed. Proc. 38, 2528-2534.
10. Bollag, W. (1983) Lancet i, 860-863.
11. Kung, W.M., Geyer, E., Eppenberger, U., and Huber, P.R. (1980) Cancer Res. 40, 4265-4269.
12. Sani, B.P., and Banerjee, C.K. (1978) Biochem. J. 173, 643-649.
13. Levine, N., Queen, L., Chalom, A.A., and Daniels, L.J. (1982) J. Invest. Dermatol. 78, 191-193.
14. McCormick, A.M., Napoli, J.L., and DeLuca, H.F. (1980) Methods Enzymol. 67, 220-233.
15. Kelly, M.A., Chandler, S.K., Alberts, D.S., Meyskins, F.L., and Haussler, M.R. (1983) J. Natl. Cancer Inst., submitted for publication.
16. Jetten, A.M., Jetten, M.E.R., Shapiro, S.S., and Poon, J.P. (1979) Exp. Cell Res. 119, 289-299.
17. Douer, D., and Koeffler, H.P. (1982) J. Clin. Invest. 69, 277-283.
18. DeLuca, L.M. (1977) Vitam. Horm. 35, 1-57.
19. Anzano, M.A., Roberts, A.B., Smith, J.M., Lamb, L.C., and Sporn, M.B. (1982) Anal. Biochem. 125, 217-224.
20. Pavlik, E.J., van Nagell, J.R. Jr., Donaldson, E., and Hanson, M. (1982) J. Steroid Biochem. 17, 553-558.
21. Pike, J.W., Kelly, M.A., and Haussler, M.R. (1982) Biochem. Biophys. Res. Commun. 109, 902-907.