LOCALIZATION OF RETINOIC ACID-BINDING PROTEIN IN NUCLEI

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SUMMARY

Retinoic acid-binding component has been detected in the nuclei of chick embryo skin. The physicochemical properties of this macromolecule are in agreement with the properties of the retinoic acid-binding protein isolated from tissue cytosol. Although no binding protein could be detected in normal colon or lung tissue, nuclei isolated from a transplantable colon tumor and Lewis lung carcinoma contained this protein.

INTRODUCTION

The importance of retinol, retinoic acid and some of their synthetic analogs in epithelial differentiation and in inhibition of tumorigenesis is well established (1-3). The molecular action of retinoids is not yet known, although there is growing evidence that they may act through cellular binding proteins. A cellular protein which specifically binds retinol has been detected and characterized (4,5). The specific retinoic acid-binding protein (RABP) originally detected from chick embryo skin has been found to be present in many tissue cytosols of rat, mouse and chick embryo (6-8). The ability of various analogs of retinoic acid to bind to RABP correlates with their biological activity in the production of mucous metaplastic cells in chick embryo skin in organ culture (9).

As an initial event in its action, retinoic acid may complex with RABP in the cytosol of the target tissues. Subsequently one would expect this complex to migrate to the nucleus of the target cell to associate with

Abbreviations: RABP, retinoic acid-binding protein; PBS, 30 mM sodium phosphate, pH 7.2 plus 100 mM NaCl; N-buffer, 50 mM Tris-HCl, 25 mM KCl, 2 mM $MgCl_2$, pH 7.5 containing 0.5 M sucrose; BSA, bovine serum albumin; OVAL, ovalbumin.

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nuclear components or nuclear acceptor sites. This nuclear localization of the receptor-ligand site is thought to stimulate transcription and thus initiate the sequence of biochemical events leading to the overall physiological changes produced by that hormone (10,11). In a study of this sequence of events, we have now demonstrated the localization of a binding component of retinoic acid in the nuclei of chick embryo skin and transplantable murine tumors.

MATERIALS AND METHODS

11,12[^{3}H]Retinoic acid (1.28 Ci/mmole), unlabeled retinoic acid, retinol and retinal were prepared by Hoffmann-La Roche, Inc., Nutley, New Jersey, and supplied to us by the Lung Cancer Branch of the National Cancer Institute. Immune γ-globulins from specific antisera to chick serum albumin and to mouse serum albumin produced in goat were purchased from Cappel Laboratories, Inc., Downington, PA.

Preparation of Nuclear Extracts and Cytosols

Skins from 12- to 13-day old chick embryos were removed and rinsed with 0.9% sodium chloride. Trocar fragments (20 mg) of colon tumor 26 and Lewis lung carcinoma, now in serial passage, were implanted subcutaneously into the axillary region of BALB/C or BDF_1 mice. The tumors developed at the primary site were removed on the 14th day after implantation. Normal colon and lung tissues were also removed from the same strain of mice.

The tissues or tumor were minced with scissors and suspended in 3 volumes (v/w) of either 30 mM sodium phosphate, pH 7.2 plus 100 mM NaCl (PBS) or 50 mM Tris-HCl, 25 mM KCl, 2 mM $MgCl_2$ pH 7.5 containing 0.5 M sucrose (N-buffer), and homogenized using a VirTis teflon homogenizer. The PBS homogenate was centrifuged at 100,000 x g for 1 hr and the supernatant used for cytosol. The N-buffer homogenate was diluted with an equal volume of the same buffer and filtered through six layers of cheese cloth to remove the cellular debris and centrifuged at 800 x g for 10 min. The pellet was resuspended and rehomogenized in 4 vols of N-buffer and centrifuged at 800 x g for 10 min. The nuclear pellet was then suspended in 20 volumes of the same buffer which had 2 M sucrose and was homogenized by several strokes with the teflon homogenizer. This suspension was centrifuged at 25,000 x g for 30 min to pellet nuclei, which were found to be homogeneously purified under microscopic examination. The nuclear pellet was then extracted with 0.3 M KCl, 0.01 M Tris-HCl, pH 7.5, 1 mM EDTA and centrifuged at 100,000 x g for 1 hr. The extract was dialyzed against PBS before use.

Albumin is known to bind retinoic acid (9,12). In order to remove traces of albumin that might be present in these extracts as a contaminant, aliquots of extracts (5 mg protein) in 0.5 ml of PBS were interacted with immune γ-globulins (2 mg protein) from specific antisera against albumin of chick serum or mouse serum as described earlier (9).

RABP was analyzed by means of sucrose gradient sedimentation (9). Portions of extracts in 0.3 ml were incubated with 300 pmoles of 11,12-[^{3}H]retinoic acid in 3 µl of dimethylsulfoxide. After dialysis of the

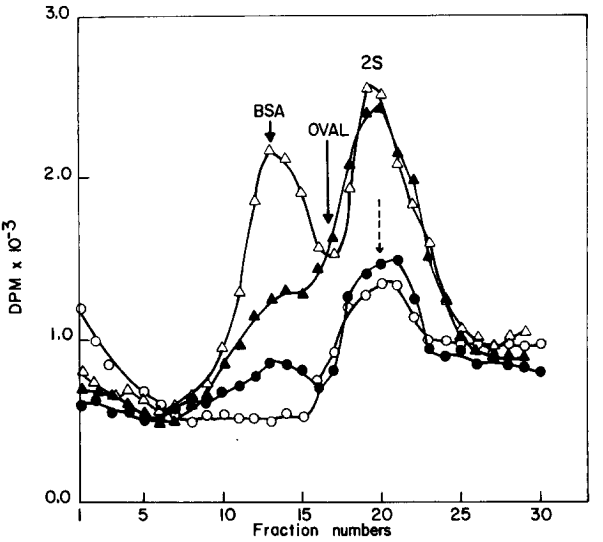

Figure 1. Sucrose gradient sedimentation patterns of (a) ●, 0.6 mg protein of chick embryo skin nuclear extract + 300 pmoles [^{3}H]retinoic acid after incubation as described in the text; (b) ○, the same as (a) except that the extract was reacted with immune γ-globulins against chick serum albumin as described in text; (c) ▲, and (d) △, 2 mg protein of chick embryo skin cytosol + 300 pmoles of [^{3}H]retinoic acid with and without, respectively, immunoprecipitation using immune γ-globulins against chick serum albumin. BSA, Bovine serum albumin; OVAL, ovalbumin.

incubation mixtures, they were centrifuged on linear 5-20% (w/v) sucrose gradients for 18 hrs at 180,000 x g in a Spinco SW 50.1 rotor at 4°. The gradients were fractionated from the bottom and the 2S peaks were located. Bovine serum albumin (BSA) and ovalbumin (oval) were used as external markers. In competition experiments, a 60-fold molar excess of the unlabeled compound in 5 µl of dimethylsulfoxide was added to the incubation mixture along with [^{3}H]retinoic acid.

RESULTS AND DISCUSSION

Figure 1 illustrates the sucrose density gradient patterns of chick embryo skin nuclear extract and cytosol fraction. The peak (dotted arrow) on the radioactivity profile of the nuclear extract showed an $S_{20,w}$ value of 2.0 which is the same as shown by the cytosol component (6,9). Like the RABP from cytosol (9), the nuclear component-[^{3}H]retinoic acid complex was digestible on incubation with Pronase, thus illustrating its protein character. Sucrose density sedimentation studies after incubation of the

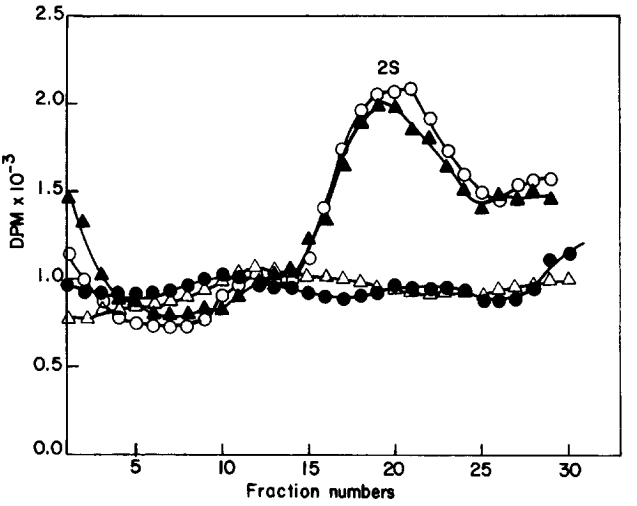

Figure 2. Sucrose density gradient centrifugation patterns: (a) Δ , (b) ● , (c) ▲ , and (d) O , are 1 mg protein of nuclei extract from normal colon, normal lung, colon tumor 26 and Lewis lung carcinomas respectively, after incubation with 300 pmoles of [^{3}H]retinoic acid as described in the text.

nuclear or cytosol fractions with high or low salt concentrations, as reported for estrogen receptors (13), did not show any altered sedimentation coefficients. As with steroid hormone receptors, RABP is found primarily in nuclei and cytoplasm, very little of this protein has been detected in the mitochondrial and microsomal fractions.

In normal mouse or rat colon and lung, RABP was below the limits of detection, whereas cytosol from mouse colon tumor 26 or Lewis lung carcinoma contained RABP in large quantities (8). Figure 2 illustrates the radioactivity profile of the nuclear extracts of the above two tumors and their corresponding normal tissues. Whereas normal colon or lung nuclei did not show any detectable amount of RABP, the extracts of nuclei from both colon and lung tumors had high levels of this protein. The mechanism by which RABP synthesis is activated during tumorigenesis is at present not understood. Other proteins are known to be present in malignant tissues or transformed cells, but absent in normal tissues (14).

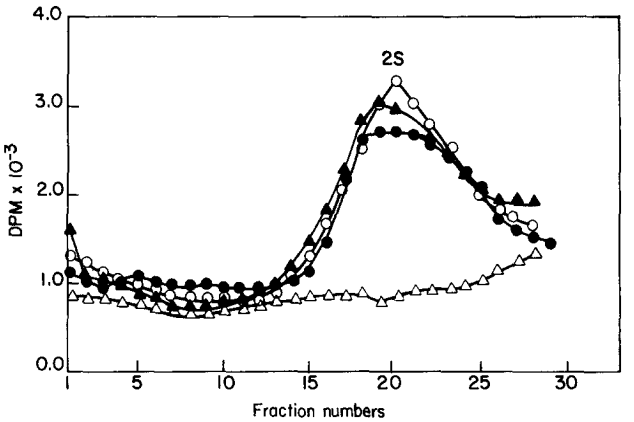

Figure 3. Sedimentation patterns of 2 mg protein of nuclear extract (after immunoprecipitation as described in the text) from colon tumor 26 plus 300 pmoles [^{3}H]retinoic acid with or without 60-fold molar excess of unlabeled test compounds (a) ▲ , nuclear extract + [^{3}H]retinoic acid; (b) Δ , (a) + unlabeled retinoic acid; (c) O , (a) + unlabeled retinol; (d) ● , (a) + unlabeled retinal.

In order to define more accurately the nuclear RABP as an intranuclear molecule, the nuclei preparation was further washed with 0.25% Triton X-100 in N-buffer and sedimented through 2.2 M sucrose. Extraction of this preparation with 0.3 M KCl resulted in lysis of nuclei (15) and release of RABP, whereas similar treatment with PBS did not show any significant amount of protein in the extract.

The ligand specificity of nuclear RABP was assessed by challenging [^{3}H]retinoic acid binding with 60-fold molar excesses of unlabeled retinoic acid, retinol and retinal (Fig. 3). With retinoic acid, the 2S peak was virtually eliminated, whereas the other competing compounds were without any effect. Binding of [^{3}H]retinoic acid is not diminished on competition with γ-linolenic acid as well. This confirms that the nuclear binding component shares similar properties in ligand specificity with the cytosol binding protein (9). We have shown that the biologically active synthetic analogs of retinoic acid such as 13-cis-retinoic acid, cyclopentenyl- and trimethylmethoxyphenyl analogs of retinoic acid, were good binders to

cytosol RABP (9). By competition experiments (not shown), they were observed to be efficiently bound to the nuclear component as well.

Although the exact nature and function of RABP-retinoic acid complex is not known, the present studies indicate no apparent difference in the physicochemical properties of the nuclear and the cytosol-binding components. There is as yet no evidence for involvement of the cytosol-binding component in the regulation of transport of retinoic acid or RABP-retinoic acid complex into the nucleus. Therefore, one can only speculate as to the biological relevance of the nuclear localization of RABP. Demonstration of the presence of nuclear RABP may be the first step in delineating the molecular mechanism of retinoic acid action for gene activation in differentiation.

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