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# RETINOIC ACID-BINDING PROTEIN: A PLASMA MEMBRANE COMPONENT

Brahma P. Sani
Kettering-Meyer Laboratory
Southern Research Institute
Birmingham, AL 32505

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## Summary

Soluble protein extracts from lyophilized plasma membranes prepared from chick embryo skin and transplantable murine carcinomas were found to contain a specific retinoic acid-binding component. This binding component showed high affinity for biologically active analogs of retinoic acid. The plasma membrane component exhibited similar physicochemical properties to those of the retinoic acid-binding protein described earlier in the cytosol. The protein exhibited mercurial-sensitive thiol functions in ligand binding; the mercurial-inhibition was reversed on treatment with thiol compounds. The plasma membrane binding component may be involved in the cellular uptake of retinoic acid.

## Introduction

Retinoids have been shown to display distinct roles in epithelial differentiation and in chemoprevention of cancer (1-4). It is generally believed that the biological action of retinoids is mediated by cellular binding proteins in a manner similar to steroid hormone (5-8). In fact, the retinoic acid-binding protein (RABP) (6) that specifically binds retinoic acid and its biologically active synthetic analogs (6,9,10) has been shown to mediate the nuclear transmigration of retinoic acid in different systems (7,8,11). The binding protein that is present in most of the retinoid-responsive epithelial tissues has also been reported in many experimental and human tumors (12,13).

Relatively little is known on the plasma transport of retinoic acid except that it is bound to serum albumin like many other fatty acids (9,10,14). The mechanism of transport of retinoic acid across the plasma membrane and its cellular uptake by epithelial cells is not well understood. In the course of a study of the special surface membrane properties of epithelial cells, we have located and partially characterized a specific retinoic acid-binding component in the plasma membrane of chick embryo skin and of some experimental murine carcinomas.

Abbreviations: RABP, retinoic acid-binding protein; CMPS, p-chloromercuriphenyl sulfonic acid.

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## Materials and Methods

Retinol, retinal, retinoic acid, phenyl analog of retinoic acid, 13-cis-retinoic acid and 11,12 [$^3$H] retinoic acid (1.12 Ci/mmole) were gifts from Hoffmann-La-Roche, Nutley, N.J. Pronase, gamma-linolenic acid, dithiothreitol and p-chloromercuriphenyl sulfonic acid (CMPS) were purchased from Sigma Chemical Company, St. Louis, MO.

Colon tumor 26 and Lewis lung carcinoma were implanted subcutaneously into BALB/c or $BDF_1$ mice (13); approximately 1-gram tumors developed at the primary site on the 16th day of implantation. Normal mouse colon and lung tissues were used as reference tissues in these studies. Epithelial layers of skin from 12- to 13-day old chick embryos were also used.

### Preparation of Plasma Membrane Extract

Plasma membrane was prepared according to the published procedure of Aronson and Touster (15) with minor modifications. The tissues were homogenized in 3 volumes of 0.25 M sucrose, filtered through 6 layers of cheese cloth, and centrifuged at 1000 x g for 10 min. The supernatant fluid was further centrifuged at 33,000 x g for 7.5 min. The supernatant solution, including the fluffy layer loosely packed on the top of the pellet, was collected by aspiration and centrifuged at 78,000 x g for 10 min. The pellet was homogenized with 1.5 volumes of 57% sucrose per gram of original tissue and placed in the bottom of a cellulose nitrate tube. The homogenate was layered with a discontinuous gradient of 34% and 8% sucrose solution and centrifuged at 25,000 rpm for 16 hr in a SW 25-2 rotor. The plasma membranes that focused at the junction of the 34% and 8% sucrose were collected and diluted with 5 volumes of distilled water and centrifuged at 100,000 x g for 60 min. The pellet was suspended in 0.03 M sodium phosphate, pH 7.2 in 100 mM NaCl and lyophilized to a dry powder. The powdered membrane was homogenized in distilled water using a VirTis teflon homogenizer and centrifuged at 100,000 x g for 60 min. The supernatant was used as plasma membrane-soluble protein extract. Fifty gramss (wet weight) of the tissues yielded 1.0-2.0 mg of the extractable protein.

Retinoic acid-binding to macromolecules was studied by sucrose density gradient sedimentation (9). Portions of the extract in 0.3 ml of 0.03 M sodium phosphate, pH 7.2 plus 100 mM NaCl were incubated with 300 pmoles [$^3$H] retinoic acid in 3 ul dimethyl sulfoxide. These mixtures, after dialysis, were centrifuged on linear 5-20% (w/v) sucrose gradients for 18 hr at 180,000 x g and fractionated (9). RABP peaks corresponding to 2S values and other peaks were located from the radioactivity profiles using bovine serum albumin and ovalbumin as external standards. In competition experiments, a 60-fold molar excess of the unlabeled test compound was added with [$^3$H] retinoic acid to the incubation mixture. For studies on thiol functions in retinoic acid binding, the plasma membrane extracts were preincubated with or without CMPS or dithiothreitol prior to [$^3$H] retinoic acid treatment and dialysis.

## Results and Discussion

The radioactivity profiles of plasma membrane extract from chick embryo skin showed a distinct peak with an $S_{20}$ value of 2.0 (Fig. 1), which is the same as that for cytosol RABP (9). Unextracted plasma membrane, suspended in 0.03 M sodium phosphate, pH 7.2 plus 100 mM NaCl, did not reveal any detectable 2S binding peak and retinoic acid bound to membrane sedimented to the bottom of the gradient. This result indicates that the binding protein is an integral part of the membrane. Plasma membrane extracts from colon tumor 26 (Fig. 1) and Lewis lung tumor (not shown) also exhibited pronounced 2S radioactive

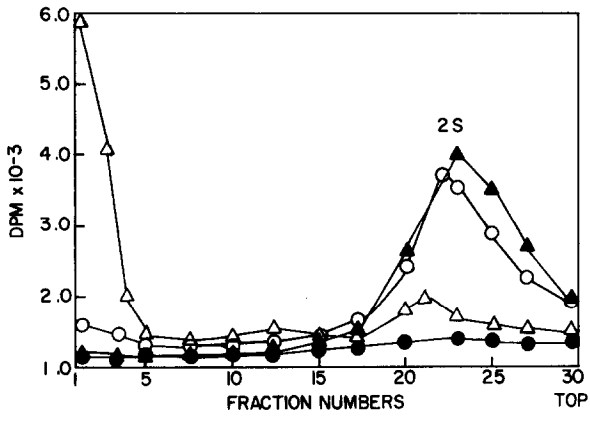


Figure 1. Sucrose density gradient sedimentation patterns of plasma membrane preparations plus 300 pmoles [$^3$H] retinoic acid. (A) △, plasma membrane suspension from chick embryo skin (1 mg protein) before lyophilization and extraction; (B) ▲, plasma membrane extract (200 ug protein) from chick embryo skin; (C) ○, plasma membrane extract (200 ug protein) from colon tumor 26; (D) ●, same as (B) except that the preparation was treated with 50 ug pronase for 60 min at 20° before dialysis and sucrose gradient sedimentation.

peaks. Lyophilization of the membrane prior to extraction was essential for maximal release of the binding component. The protein nature of the binding component in the extracts was assessed by treatment with pronase, which completely digested the 2S component (Fig. 1). No detectable amounts of RABP, as measured from the 2S radioactive peak, was extracted from adult mouse lung or colon plasma membranes.

It is known that a retinoic acid-like structure with a ring, side chain and free terminal carboxyl group is essential for maximal binding to cytosol RABP (6,9,10). A similar requirement was also found to be necessary for binding to plasma membrane RABP. Ligand specificity of plasma membrane RABP was assessed by challenging [$^3$H] retinoic acid with 60-fold molar excess of unlabeled test compounds (Fig. 2). A 60-fold excess of gamma-linolenic acid did not show any competitive effect, whereas a similar excess of unlabeled retinoic acid virtually eliminated the 2S peak. Under similar conditions, retinol or retinal showed no effect at all (not shown). We have shown earlier that a correlation exists between the binding affinities of retinoic acid analogs to cytosol RABP (6,9,10) and their biological potency in epithelial differentiation and antitumorigenesis (3,16,17). The present studies indicate that the phenyl analog of retinoic acid, which has negligible biological activity (16,17), is a poor competitor for retinoic acid-binding sites on plasma membrane

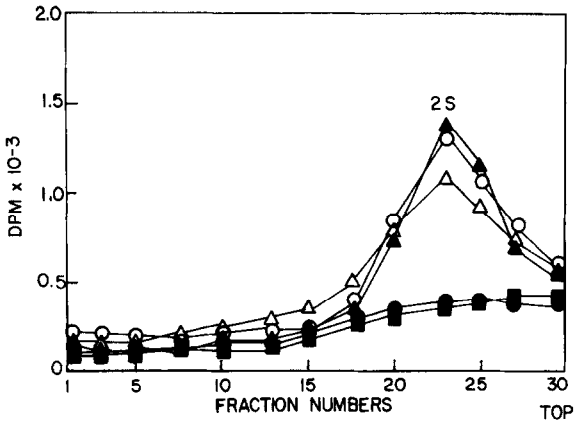


Figure 2. Sedimentation patterns of 200 ug protein of plasma membrane extract from chick embryo skin plus 300 pmoles [$^3$H] retinoic acid with or without 60-fold molar excess of unlabeled test compounds. (A) ▲, plasma membrane extract + [$^3$H] retinoic acid; (B) ■, (A) + unlabeled retinoic acid; (C) ●, (A) + 13-cis-retinoic acid; (D) △, (A) + phenyl analog of retinoic acid; (E) ○, (A) + gamma-linolenic acid.

(Fig. 2). On the other hand, 13-cis-retinoic acid which has pronounced in vivo anticarcinogenic property as well as high biological potency in epithelial differentiation (16-18), competed as efficiently as retinoic acid for the binding sites (Fig. 2). Thus the general pattern in the affinities for binding to the plasma membrane RABP by retinoic acid and its synthetic analogs is similar to their affinities for cytosol RABP (6,9,10).

We examined whether or not functional thiol groups played a role in retinoic acid binding to plasma membrane component. Figure 3 illustrates the sucrose gradient sedimentation patterns of 200 ug protein of plasma membrane extract with bound [$^3$H] retinoic acid after exposure to CMPS or dithiothreitol. The mercurial treatment completely inhibited the [$^3$H] retinoic acid binding capacity of the protein. Such a mercurial-inhibited preparation was further treated with 5 mM dithiothreitol followed by incubation with [$^3$H] retinoic acid. About 90% retinoic acid-binding capacity was recovered, indicating the reversible nature of ligand-binding.

The physicochemical properties of plasma membrane RABP so far studied indicate that they are similar to cytosol RABP (6,9,19). Thus they exhibit similar sedimentation constants, ligand specificity, functional groups (this report) and isoelectric pH (4.5) (not shown). Similar to cytosol RABP (13), the plasma membrane binding component also is present in large quantities in the embryonic tissues, marginally detectable in adult tissues,

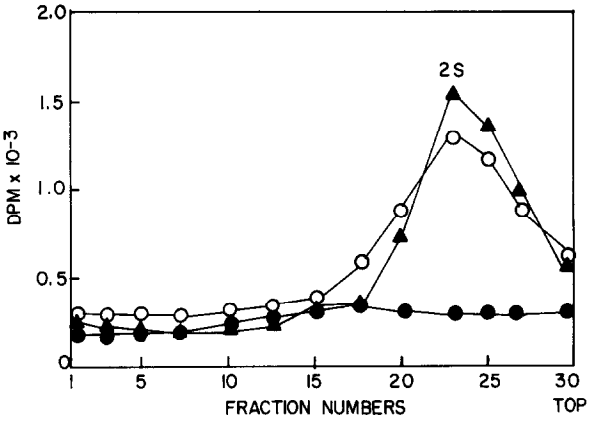


Figure 3. Sucrose gradient sedimentation patterns of CMPS-treated (1 hr, $4^{o}$) plasma membrane extract (200 ug protein) from chick embryo skin plus [$^{3}$H] retinoic acid. (A) ▲, control with no CMPS; (B) ●, 2 mM CMPS; (C) ○, same as (B) except that it was further incubated with 5 mM dithiothreitol at $4^{o}$ for 1 hr before incubating with [$^{3}$H] retinoic acid.

but evident after neoplastic transformation. The fact that the phenyl analog of retinoic acid does not bind to the plasma membrane component suggests that this retinoid may not be transported across the cell surface membrane and therefore tends to be biologically inactive for cellular functions. In contrast, the high binding affinity of the binding component for 13-cis-retinoic acid may be required for selective transport of this biologically active retinoid. Since we already know that cytosol RABP may mediate the nuclear entry of retinoic acid (7,8,11), the demonstration of the existence of plasma membrane RABP may be yet another critical step in the delineation of the molecular mechanism of action of retinoic acid in differentiation and tumorigenesis.

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