DEMONSTRATION OF A NOVEL MOLECULAR SPECIES IN CHICK EMBRYO BRAIN: CELLULAR RETINOL-BINDING PROTEIN, F-TYPE

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Summary By sucrose density gradient sedimentation analysis, a cytosol component of small molecular size which bound \(^3\)H-retinoic acid was detected in the chick embryo brain, exhibiting a peak on day 14 (7 days before hatching). In general, cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP) have been distinguished by showing binding specificity for retinol or retinoic acid, respectively. However, the component found in embryo brain cytosol exhibited binding affinity for both retinol and retinoic acid, being similar in less ligand-specificity to CRBP (F) which was originally recognized in fish eye cytosol. Moreover, the component also showed a lack of binding affinity for prealbumin (PA), excluding the possibility that chicken plasma RBP was contaminated in the cytosol. These findings strongly suggest that a distinct molecular species of cellular binding protein for vitamin A exists in the brain cytosol of a developing chick embryo.

Keywords vitamin A, retinol, retinoic acid, cellular retinol-binding protein, cellular retinoic acid-binding protein, ligand specificity, onco-fetal protein

Recently, it has become well established that a cytosol-binding protein specific for retinol (CRBP) is distinct from the plasma retinol-binding protein (RBP), and widely exists in several rat tissues (1), rabbit (2), cow (3) and chick embryo (4). Recently, it has been reported that CRBP, extensively purified from rat liver (5) and testis (6) to a homogeneous component, has a molecular weight of approximately 14,600, and binds retinol exclusively on a 1:1 molar basis.

On the other hand, a cytosol-binding protein highly specific for retinoic acid

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Abbreviations: RBP, retinol-binding protein; PA, prealbumin; CRBP(F), cellular retinol-binding protein, fish-type; CRABP, cellular retinoic acid-binding protein.
(CRABP) has also been detected in a limited number of tissues (i.e., the reproductive organ, eye and brain) of the rat (7), the bovine retina (8) and the chick embryo skin (9). Moreover, Ong and Chytíl (10) have recently demonstrated that CRABP, purified from rat testis, has an identical molecular weight, 14,600, to that of rat liver CRBP, but specifically binds retinoic acid on a 1:1 molar basis.

Shidoji and Muto (11) have recently reported that in addition to CRABP, fish eye cytosol contains a distinct binding protein with affinity for both retinol and retinoic acid, that is designated cellular retinol-binding protein, fish-type or CRBP (F). During the course of our studies on vitamin A metabolism in relation to specific binding proteins of chick embryo (12), we have incidentally observed a cellular binding component in the chick embryo brain which is similar in molecular size and ligand-specificity to CRBP (F).

MATERIALS AND METHODS

Fertilized eggs of the white leghorn (Thornber) were incubated in a hatching incubator at 38°C in 80% humidity. Brains, at several embryonic stages and after hatching, were carefully removed and weighed. The brain was immediately homogenized with 2 volumes of 50 mM Tris-HCl, pH 7.6, 25 mM KCl and 5 mM MgCl₂ (designated TKM buffer) by a glass-Teflon homogenizer. The supernatant (7,800 g, 10 min) was then centrifuged at 105,000 g for 60 min, and the resulting cytosol was used immediately for assays or stored at -20°C until needed. Immunoassays of both chicken plasma RBP and PA (12) were done on the brain cytosol which was obtained from the original homogenate pretreated with or without 0.5% Nonidet P-40 (Shell Chemical Co.) by centrifugation at 15,000 g for 30 min. In order to eliminate plasma RBP, the brain cytosol was subjected to affinity chromatography on a column of human PA-coupled Sepharose according to the method of Vaňhquist et al. (13); the specific details have been described previously elsewhere (14).

The cytosol specimens were incubated with all-trans [11,12-3H] retinoic acid (11.1 Ci/m mole, Hoffmann-LaRoche) with or without a molar excess of either unlabeled all-trans retinoic acid (Hoffmann-LaRoche) or all-trans retinol (Sigma Chemical Co.). After incubation at 4°C, each sample was subjected to either ultracentrifugation on a linear 5 to 20% sucrose density gradient in TKM buffer or gel filtration on Sephadex G-75 (Pharmacia Fine Chemicals) equilibrated with 50 mM Tris-HCl, pH 7.4, 300 mM KCl and 1 mM dithiothreitol (designated TKD buffer). Bovine serum albumin (Nutritional Biochemicals Corp.) and myoglobin (Schwarz-Mann Co.) were employed for sucrose density gradient sedimentation analysis as external standards. Specific details of typical illustrative examples are given in the legends to the appropriate figures (Figs. 2 and 3). Radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3380. In general, 6 ml of a scintillation cocktail (Triton X-100: toluene: Omnifluor; 333 ml:
RESULTS AND DISCUSSION

Changes in levels of cellular binding protein labeled with $^3$H-retinoic acid during embryonic development of the chick brain

As previously reported from this laboratory (12), immunoreactive plasma RBP and PA were also found in the chick embryo brain. When both RBP and PA levels were expressed on a basis of per mg cytosol protein, the two proteins exhibited a peak on day 6 (15 days prior to hatching), being consistent with the previous observation (12). However, the time course of changes in the total contents of RBP and PA were found to be different each other, as shown in Fig. 1. On embryonic day 14 (7 days before hatching), the brain cytosol contained three times more PA (mol. wt: 55,000) than RBP (mol. wt. 20,000), suggesting an apparent 1:1 molar complex formation similar to that in native plasma. Additional study revealed that
Fig. 2. Demonstration of CRBP (F) in the brain cytosol of 14-day-old chick embryo. Sucrose density gradient centrifugation patterns. Each 200 μl of the brain cytosol (1.25 mg protein) was incubated at 4°C for 4-8 hr with 1 μl of 3H-retinoic acid (1.26 × 10^4 dpm, 8 pmoles) dissolved in ethanol to give a final concentration of 400 nM (●). Displacement of the radioactivity was done by simultaneously adding a 150-fold molar excess of unlabeled retinoic acid (×××) or retinol (○○○). Each sample (0.2 ml) was then layered on a linear 5 to 20% sucrose gradient dissolved in TKM buffer (4.8 ml) and centrifuged at 178,900 g for 20 hr using an Hitachi 65P (RPS 65TA rotar). After centrifugation, fractions of about 0.2 ml each were collected in counting vials, to which 0.3 ml of distilled water and 5 ml of a scintillation solution were added for measurement of radioactivity. Each 0.2 mg of bovine serum albumin (BSA) and myoglobin (Mb), dissolved in 0.2 ml of TKM buffer were simultaneously run, as indicated by the arrows in the figure.

immunoreactive RBP and PA were almost quantitatively recovered in the cytosol without previous treatment of Nonidet P-40 for solubilization.

By sucrose density gradient sedimentation technique, a cellular binding component labeled with 3H-retinoic acid was detected which had a small molecular size and a low capacity; complete displacement of radioactivity was obtained by adding a 150-fold molar excess over the radioisotope. In sharp contrast to RBP and PA, however, the component in the whole brain exhibited a peak on day 14 (7 days before hatching), as shown at the bottom of Fig. 1. This result is compatible with the observation made by ONG and CHYTIL (15) that CRABP of the rat embryo (i.e., liver, lung etc.) decreased and finally disappeared during perinatal development.

Ligand specificity of the cellular binding protein in chick embryo brain: The presence of CRBP (F)

Very little is known about CRABP of the chick brain and its perinatal
Fig. 3. Demonstration of CRBP (F) in the brain cytosol of 14-day-old chick embryo: Elution profiles on gel filtration of Sephadex G-75. Seven milliliters of the cytosol was initially applied to a column (0.9 × 6.5 cm) of human PA-coupled Sepharose. The peak 1 (12 ml), which had no affinity for human PA, was dialyzed twice against distilled water. After lyophilization for 7 hr, the dry material was dissolved in 3.5 ml of TKM buffer. Each 400-μl sample was incubated with 3H-retinoic acid (••• ) at a concentration of 200 nM for 16 hr at 4°C. Displacement of the radioactivity was done by simultaneously adding a 200-fold molar excess of either unlabeled retinoic acid (××× ) or retinol (○○○ ). After incubation, each sample was subjected to a column (0.9 × 83 cm) of Sephadex G-75 equilibrated with TKD buffer. Fractions of 1.5 ml each were collected at a flow rate of 13 ml/hr for counting the radioactivity. The large radioactive peak which corresponds to free 3H-retinoic acid is deleted in the figure.

dev elopment, with the exception of the previous observation reported by SANI and CORBETT (16). However, no definite statement was made by these authors with regard to ligand specificity of the CRABP in chick embryo brain.

Of great interest is the finding that 3H-retinoic acid bound to a cytosol component of small molecular size (similar to that of myoglobin; mol. wt. 17,800) was displaced not only by unlabeled retinoic acid, but also by retinol at a 150-fold molar excess over the radioisotope by sucrose density gradient centrifugation (Fig. 2). This unexpected result strongly suggests that brain cytosol contains a
different binding protein(s) from either CRABP or CRBP, in terms of ligand specificity.

As already reported (11), no binding components specific for either retinol or retinoic acid were seen, when native plasma (i.e., a tight complex formation between RBP and PA) was incubated with the labeled vitamins without further purification or pretreatments by organic solvents. Thus, no significant contribution of immunoreactive RBP available in the cytosol is conceivable. However, an attempt was made to eliminate the immunoreactive plasma RBP from the cytosol by human PA-affinity chromatography, and then to examine whether or not a new molecular species of cellular binding protein still remains. As shown in Fig. 3, elution profiles of gel filtration on Sephadex G-75 clearly demonstrated the presence of a cytosol-binding protein with affinity for both retinoic acid and retinol. Moreover, almost all of the radioactivity bound to the specific component (combined fractions of 26 to 31 on the gel filtration, Fig. 3) was found to be recovered at the peak 1 (without any affinity for PA), when reexamined by the human PA-affinity chromatography. Preliminary experiments further disclosed that the cytosol component was also labeled with $^3$H-retinol, and eluted slightly later than CRABP by gel filtration on a longer column of Sephadex G-75, Superfine: the binding protein is assumed to have a smaller molecular size than that of CRABP (Data not shown). In any event, these results strongly suggest the presence of a distinct binding protein in the chick embryo brain which is compatible with CRBP(F) originally recognized in the fish eye cytosol, with special regard to less ligand specificity (11). Needless to say, further studies are obviously required to isolate and characterize this new molecular species of CRBP(F).

Of additional evidence are the findings in this laboratory that a similar molecular species to CRBP(F) was also detectable in human fetal liver, at a certain phase of regenerating rat liver, and in human hepatocellular carcinoma at much higher levels than those of CRABP (MUTO and OMORI*). Therefore, it is conceivable that CRBP(F) is an ancient protein in phylogenesis as well as an onco-fetal protein in nature. Biological and clinical roles of the protein in cell differentiation and carcinogenesis warrant further exploration.

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REFERENCES


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