Cellular retinoid-binding proteins in regenerating rat liver: demonstration of a novel cellular retinoid-binding protein

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Abstract Changes in the levels of liver cellular retinol- and retinoic acid-binding proteins were studied after partial (about 70%) hepatectomy for 14 days in the rat. It was found that a novel binding protein designated F-type appears transiently in liver cytosol 3 days after the operation. The appearance of this protein coincides with the peak level of the α_1-fetoprotein. In contrast, cellular retinoic acid-binding protein was detected only the first day after hepatectomy, whereas no significant change was observed in the level of the cellular retinol-binding protein during the entire observation period. [^{3}H]Retinol or [^{3}H]retinoic acid complexed with serum retinol-binding protein injected intravenously into vitamin A-deficient rats 1 day after the hepatectomy was recovered 5 min or 20 min later bound specifically to cellular retinol- or retinoic acid-binding protein, respectively. The results presented here strongly suggest that each of the three cellular retinoid-binding proteins plays a distinct role in cell proliferation and differentiation.—**Omori, M., Y. Muto, and T. Nagao.** Cellular retinoid-binding proteins in regenerating rat liver: demonstration of a novel cellular retinoid-binding protein. *J. Lipid Res.* 1981. **22:** 899–904.

Supplementary key words vitamin A · retinoic acid · vitamin A-deficiency · α_1-fetoprotein

It has been established that cytosols of many tissues contain two different binding proteins for vitamin A and its analogs (retinoids). The first one is cellular retinol-binding protein (CRBP) (1) and the second is cellular retinoic acid-binding protein (CRABP) (2). CRBP (3, 4) and CRABP (5, 6) have been purified to homogeneity. Both proteins have molecular weight of 14,600 but can be distinguished by their binding specificity either for retinol or retinoic acid. Shidoji and Muto (7) have recently reported that the fish eye cytosol contains another cellular retinoid-binding protein with a similar molecular size but which appears to have binding affinity for both retinol and retinoic acid. A similar binding protein has also been detected in the brain cytosol of developing chick embryo (8), human fetal liver, and human hepatocellular carcinoma (9). We call this cellular retinoid-binding protein F-type (CRBP (F)) as suggested previously (8, 9). The present study was undertaken to determine whether the levels of these proteins change during liver regeneration.

EXPERIMENTAL PROCEDURE

Partial hepatectomy

Weanling rats (male), Sprague-Dawley (Japan Charles River Co., Atsugi City, Kanagawa), weighing 40–50 g were fed either a vitamin A-deficient diet described earlier (10) or a normal balanced stock diet (Japan Clea Inc., Tokyo) for 27 days. The rats were then anesthetized with diethyl ether and about 70% of the liver was removed between 10:00 and 11:00 AM. The percentage of regeneration was determined from the recovery of liver weight as previously described (11).

Determination of cellular retinoid-binding proteins

Liver specimens were used immediately for assay or stored at −20°C until needed. The assays were carried out as previously described (9). Briefly, liver cytosol (105,000 *g*, 60 min) was incubated in the dark for 16 hr with either all-*trans* [15-^{3}H]retinol (2.66

Abbreviations: CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; CRBP(F), cellular retinoid-binding protein, F-type; RBP, serum retinol-binding protein; PA, prealbumin; AFP, α_1-fetoprotein; PAGE, polyacrylamide gel electrophoresis.

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Ci/mmol; New England Nuclear, Boston, MA) for CRBP or all-*trans* [11,12-^{3}H]retinoic acid (11.1 Ci/mmol; Hoffman-LaRoche, Nutley, NJ) for CRABP determination to give a final concentration of 200 nM. Simultaneously, unlabeled all-*trans* retinol (Sigma Chemical Co., St. Louis, MO) or retinoic acid (Hoffmann-LaRoche) was added in a 200-fold molar excess over the radioactive compound to measure the specific binding. Then, the amount of the labeled ligands bound to the particular cellular retinoid-binding protein was assessed by gel filtration on Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala). Each cellular binding protein was identified in the elution volume of radioactive peak corresponding to molecular size of co-chromatographed piscine serum retinol-binding protein (mol wt 16,000) (12). The binding activity was expressed in pmol of the ligand specifically bound per gram of tissue by subtracting the radioactive counts recovered in the absence and presence of an excess of unlabeled ligands. The level of CRBP(F) was assessed by determining the difference of radioactivity recovered from the gel filtration column after incubating the cytosols with labeled retinoic acid alone and with labeled retinoic acid in the presence of the 200-fold molar excess of retinol.

Partial purification of cellular retinoid-binding proteins

CRBP, CRABP, and CRBP(F) were partially purified from the liver removed during hepatectomy and from livers obtained 24 hr and 72 hr after the hepatectomy, respectively. Each cytosol (about 3–5 ml), containing 0.1 mM phenylmethanesulfonyl fluoride (Sigma) was incubated with [^{3}H]retinol (200 nM) or [^{3}H]retinoic acid (80 nM) at 4°C for 16 hr. Each sample was first applied to a column (0.9 × 6.5 cm) of human prealbumin (PA)-coupled Sepharose (13) to eliminate serum retinol-binding protein RBP (12). Then, the eluate (Peak 1) (12) was chromatographed on a column (1.9 × 65.0 cm) of Sephadex G-75, Superfine (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl. Fractions corresponding to a molecular size of about 15,000 were pooled and dialyzed exhaustively against distilled water. After subsequent lyophilization, each sample was dissolved in 0.5–1.0 ml of the above buffer. This preparation was characterized further.

Enzyme treatment of partially purified CRBP(F)

Binding ability of the partially purified CRBP(F) to radioactive retinoic acid was examined after treatments with enzymes such as pronase (B grade, Calbiochem, San Diego, CA), deoxyribonuclease (DNase I, Worthington Biochemical Corporation, Freehold, NJ), ribonuclease (RNase R, Worthington), neuraminidase (B grade, Calbiochem) and trypsin (Boehringer and Soehne, Mannheim). Partially purified sample obtained as described above (50 μl) was incubated with pronase, DNase, RNase, trypsin (200 μg of each in 50 μl of 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4), or neuraminidase (50 μl) at room temperature for 3 hr and then subjected to analytical PAGE. Protein-bound [^{3}H]retinoic acid was then measured by determining the radioactivity of the sliced gels, and percent of binding was compared with that obtained when no enzyme was added.

Gel electrophoresis

Disc polyacrylamide gel electrophoresis (14) and isoelectric focusing in polyacrylamide gel (15) were carried out, as previously described (12). In some experiments, partially purified CRABP labeled with [^{3}H]retinoic acid and CRBP labeled with [15-^{14}C]-retinol (10 mCi/mmol, Radiochemical Center, Amersham) were co-electrophoresed. Radioactivity and pH in the sliced gels were measured as previously reported (12).

Molecular weight estimation

Molecular weights of the partially purified binding proteins were estimated using a column of Sephadex G-75, standardized by proteins of known molecular weight. The following markers were used: bovine serum albumin, ovalbumin, myoglobin, and cytochrome C (Schwarz-Mann, Orangeburg, NY) and piscine serum retinol-binding protein previously purified in this laboratory (12). Blue dextran 2,000 (Pharmacia) and methyl green (E. Merck, Darmstadt) were also co-chromatographed.

In vivo administration of [^{3}H]retinol and [^{3}H]retinoic acid to vitamin A-deficient rats after partial hepatectomy

Labeled retinoids complexed with human RBP were injected. To this end, human apo-RBP (without bound retinol) purified from human urine of patients suffering from "Itai-Itai" disease (16) was generously provided by Dr. M. Kanai. Four nmoles of [^{3}H]retinol in 10 μl of ethanol was added to 0.9 ml apo-RBP (450 μg/ml 0.15 M NaCl). Similarly, 1 nmole of [^{3}H]retinoic acid in 10 μl of ethanol was added to 0.9 ml apo-RBP (100 μg/ml 0.15 M NaCl). After addition of [^{3}H]retinol or [^{3}H]retinoic acid, the solution was incubated at 4°C for 30 min. Column chromatography using Sephadex G-75 has shown

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that all radioactive retinol or retinoic acid was bound to RBP.

[^{3}H]Retinol-RBP complex or [^{3}H]retinoic acid-RBP complex (4.4 μCi/1.8 nmol [^{3}H]retinol–9 nmol of RBP in 400 μl or 4.4 μCi/0.43 nmol [^{3}H]retinoic acid–2.2 nmol of RBP in 400 μl) were injected into the portal vein under ether anesthesia to the vitamin A-deficient rats 1 day after hepatectomy. Five or 20 min after the injection, the liver from each rat was removed and cytosol was prepared immediately. To remove serum RBP, each cytosol was subjected to the human PA affinity chromatography. Total radioactivity was measured in an aliquot of the cytosol to determine radioactive retinoid present, and it was immediately applied to Sephadex G-75 (superfine) column, equilibrated, and eluted with 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl. Fractions of 2 ml were collected at a flow rate of 14 ml/hr. Subsequently, two aliquots were incubated with a 200-fold molar excess of respective unlabeled retinoid (based on the radioactivity recovered in the cytosols) for 16 hr at 4°C and then applied to Sephadex G-75 (superfine) column, as described above.

Other procedures

Liver vitamin A content was determined by the trifluoroacetic acid method after extraction with ether as described by Ames, Risley, and Harris (17). α_1-Fetoprotein (AFP) in the liver cytosol was measured by radioimmunoassay (18)

RESULTS

Liver vitamin A after feeding vitamin A-deficient diet

Liver vitamin A became undetectable when weanling rats were fed the vitamin A-deficient diet for 20 days.

TABLE 1. Contents of CRBP, CRABP, CRBP(F), and vitamin A in regenerating livers after partial hepatectomy in normal rats

Days after Resection	CRBP	CRABP	CRBP(F)	Vitamin A
		pmoles/g liver		*μg/g liver*
0	153.0	BD[b]	BD	92.4 ± 3.1[c]
1	85.3	5.1 ± 1.2	BD	85.8 ± 2.8
2	46.6	BD	BD	70.6 ± 2.7
3	50.3	BD	9.3 ± 2.0	58.5 ± 1.7
5	72.2	BD	BD	46.4 ± 2.1
6.5	ND[a]	BD	BD	42.4 ± 2.5
8	165.2	BD	BD	62.3 ± 2.2

[a] ND, not determined.
[b] BD, below detection.
[c] Mean ± SEM.

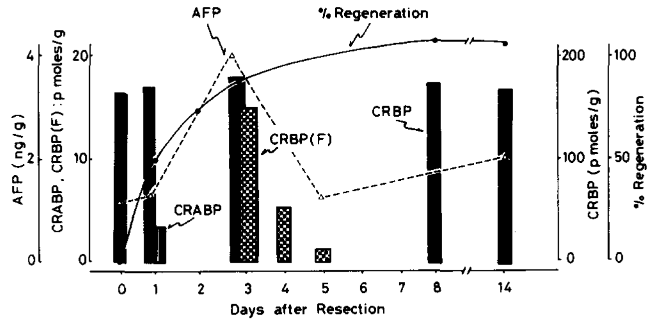

Fig. 1. Time course of changes in CRBP, CRABP, and CRBP(F) levels in regenerating livers after hepatectomy in vitamin A-deficient rats. ⊙ —— ⊙, % hepatic regeneration; △ —— △, α_1-fetoprotein.

Time course of changes in CRBP, CRABP, and CRBP(F) during hepatic regeneration

Substantial changes in the levels of the binding proteins were observed after partial hepatectomy of the normal rats (**Table 1**). Decrease of CRBP was observed between day 1 and day 5. Normal values were attained on day 8 after operation when complete regeneration was also observed. On the other hand, CRABP and CRBP(F) appeared only transiently, being detectable only 24 hr or 72 hr after the resection of the liver, respectively.

When vitamin A-deficient rats were used (**Fig. 1**), CRBP levels were little changed during 14 days after partial hepatectomy. Here again, CRABP and CRBP(F) analyzed each day after the operation appeared only transiently. CRABP was detected at 24 hr, whereas CRBP(F) appeared after 72 hr but rapidly decreased to an undetectable level at 6.5 days. AFP was also detected at the time coincident with that of CRBP(F). It is of interest that in vitamin A-deficient animals (levels of vitamin A were below detection) the liver regeneration proceeded with the speed and to the extent observed in control animals. This observation is in contrast with the findings observed previously by others (19).

Partial characterization of CRBP, CRABP, and CRBP(F) from regenerating livers

When the partially purified cellular retinoid-binding proteins were subjected to analytical PAGE, relative mobilities of CRBP and CRBP(F) were found to be the same (R_m 0.73) but greater than that of CRABP (R_m 0.69 (**Fig. 2**)). The isoelectric point of CRABP (pI 4.8) was somewhat higher than that of CRBP (pI 4.7) which was indistinguishable from that of CRBP(F). The partially purified CRBP(F) revealed an affinity for both retinoic acid and retinol that is in sharp contrast to a strict ligand-specificity of CRBP and CRABP. The partially purified CRBP(F) labeled

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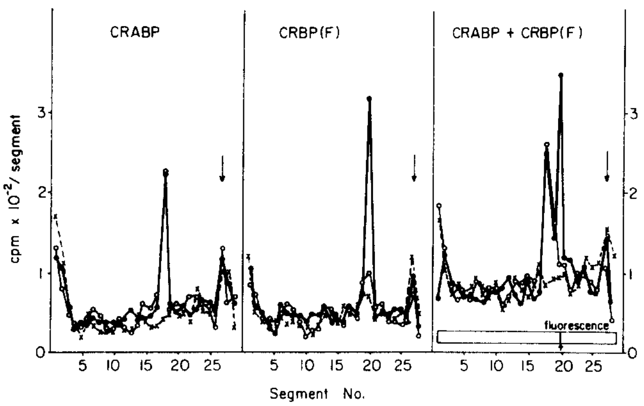

Fig. 2. Polyacrylamide gel electrophoresis of CRABP and CRBP(F). Solutions (100 μl) of partially purified CRABP and CRBP(F) labeled with [^{3}H]retinoic acid were incubated with 200-fold molar excess of unlabeled retinol (O —— O), 200-fold molar excess of unlabeled retinoic acid (X – – – X) or without any competitors (● —— ●). When a mixture of CRABP and CRBP(F) was applied on PAGE (extreme right) the fluorescence of protein-bound retinol was observed at the elution position of CRBP(F). The arrow indicates the position where tracking dye migrated.

with radioactive retinoic acid was treated with several enzymes; the binding was reduced to 6% and 15% by treatment with pronase and trypsin, respectively, indicating that the binding component was a protein. Using a standardized column of Sephadex G-75, the molecular weights of CRBP, CRABP, and CRBP(F) were found to be almost the same (about 15,000).

In vivo labeling of CRBP and CRABP with radioactive ligands

[^{3}H]Retinol or [^{3}H]retinoic acid complexed with RBP were injected into vitamin A-deficient rats 1 day after hepatectomy and 5 min later livers were removed and treated as described above. Gel filtration showed a presence of radioactive peaks eluting at the positions of CRBP or CRABP, respectively. Data from such an experiment are shown in **Fig. 3**. When cytosols were prepared 20 min after injection of the complexes, gel filtration revealed labeled peaks of CRBP and CRABP. Radioactivity bound to the cellular retinoid-binding proteins was completely displaced by adding a 200-fold molar excess of unlabeled retinoids in vitro thus indicating presence of specific binding. In the period from 5 min to 20 min after the injection, the specific binding increased 2.1-fold in CRBP and 1.8-fold in CRABP. Fractions of larger molecular size than the cellular binding proteins were components that bind retinoids nonspecifically, since no displacement was observed by addition of an excess of unlabeled ligands. Free (unbound) radioactivities were seen at the eluting position similar to that of methyl green.

DISCUSSION

The data presented here indicate that CRBP(F) detected previously in fish eyes (7), chick embryo brain (8), human fetal liver, or hepatocellular carcinoma (9) appears transiently on the third day after partial hepatectomy in control as well as in vitamin A-deficient rats. CRBP(F) of the regenerating adult rat liver has a molecular weight of 15,000 and posseses binding affinity for retinol as well as for retinoic acid. Such affinity was demonstrated by labeling the protein with retinoic acid, and displacing it by unlabeled retinol. Conversely, like CRBP(F) from other sources (7–9), the liver protein can be labeled with radioactive retinol. Here again excess of unlabeled retinoic acid can displace binding of labeled retinol (data not shown). For practical reasons we have used radioactive retinoic acid for the detection. This protein is different from CRBP which has strict specificity for retinol and whose characteristics have been reviewed recently (20). On the other hand, liver

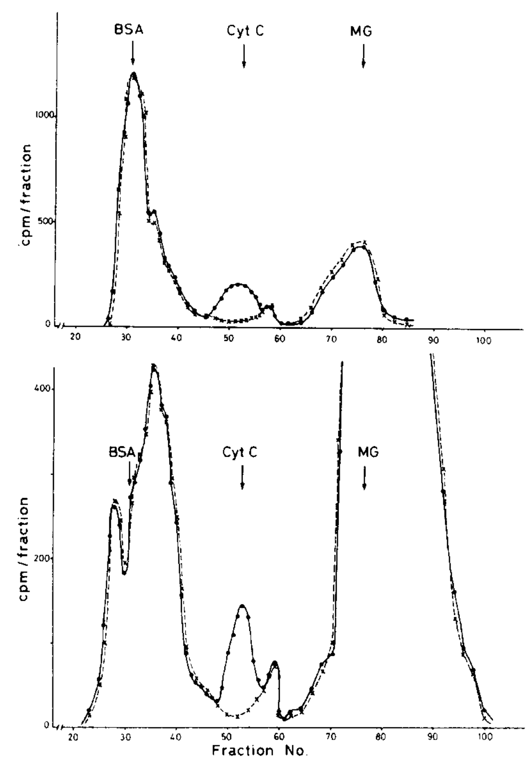

Fig. 3. Elution pattern of CRBP (top panel) and CRABP (bottom panel). Vitamin A-deficient rats 24 hr after hepatectomy were injected with [^{3}H]retinol or [^{3}H]retinoic acid complexed with RBP. Twenty min later liver cytosols were prepared and treated as described in Experimental Procedure. CRBP and CRABP were detected by gel filtration on Sephadex G-75. Cytosols were incubated without (●——●) or with (X – – – X) 200-fold excess of nonlabeled ligands. Arrows indicate the eluting positions of bovine serum albumin) (BSA), cytochrome C (Cyt C), and methyl green (MG), respectively.

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CRBP(F) has similar characteristics to liver CRBP in molecular weight, mobility on analytical polyacrylamide gel electrophoresis, and isoelectric point. When compared with CRABP which specifically binds retinoic acid and not retinol (20), CRBP(F) differs in the binding ability, mobility on polyacrylamide gel electrophoresis, and in isoelectric point, but not in the molecular weight. We have performed preliminary experiments to determine whether CRBP(F) originated from CRBP during processing. Liver tissue, homogenates, or cytosol containing CRBP(F) were mixed with those from control rats where this protein is not detectable. Augmentation of the CRBP(F) was not observed after processing of these samples. This can be taken as an indication that CRBP is not the source of CRBP(F).[2]

The transient appearance of CRBP(F) after partial hepatectomy was independent of the presence of CRBP and did not coincide with the temporary appearance of CRABP. This indicates that different mechanisms are involved in regulation of the synthesis of those proteins. That the regulation of CRBP synthesis is distinct from that of CRABP has been shown previously in experiments involving developing lung and liver (21).

This study also shows that the liver regeneration is apparently not affected by the vitamin A status, which is in sharp contrast with results published earlier (19). At present we cannot explain this discrepancy. The vitamin A status seems to have no effect on the CRBP levels before hepatectomy. In contrast, the levels of liver CRBP after hepatectomy of control rats appear to diminish temporarily and reach control levels when the livers are fully regenerated. No changes in CRBP in the livers of vitamin A-deficient rats were observed. The difference between control and vitamin A-deficient rats suggests the existence of an alternative mechanism operative in the maintenance of the levels of CRBP after hepatectomy.

The appearance of CRBP(F) coincides with time when α_1-fetoprotein appears (22), and pyruvate kinase type III (fetal type) can also be detected (23). This suggests that CRBP(F) may be an onco-fetal protein having a role in cell proliferation represented by the model of liver regeneration.

The transient appearance of CRBP(F), distinctly different from that of CRABP in the regenerating liver, also suggests the different role of these proteins in the regeneration process. CRABP has been shown to be present in fetal but not in adult livers (21). The appearance of CRABP in regenerating liver shows that this protein might be related to the rapid proliferative phase of the adult liver (24).

Finally the results presented here indicate that CRBP and CRABP can be recovered from livers of vitamin A-deficient animals when the respective ligands are injected intravenously as radioactive compounds[3] complexed with RBP.

The function of the three cellular retinoid binding proteins in the cell metabolism is not clear. Further work will be required to delineate the role of these proteins in cell proliferation.

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[2] In contrast to liver CRBP, binding activity of CRBP(F) was not inhibited after adding increasing amounts of anti-liver CRBP antibody, generously provided by Dr. F. Chytil (Omori, M. Unpublished data.).

[3] After the administration of [^{3}H]retinoic acid-RBP complex, thin-layer chromatography and alumina column chromatography showed that almost all the radioactivity in liver lipid extract was retinoic acid.

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