Purification, Properties, and Developmental Changes of Cellular Retinol-Binding Protein, Type II, in Chicken Intestine

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Summary Two distinct cellular retinol-binding proteins were detected in chicken small intestine. A predominant form was purified to homogeneity. The apparent molecular weight of this protein was estimated to be 17,200. This form was larger than a second minor form partially purified (molecular weight of 15,000). The absorption and fluorescence spectra of the bound retinol to the purified proteins were typical for the known cellular retinol-binding proteins. The results suggest that the purified binding protein corresponds to CRBP(II), previously identified in small intestine of rats and humans. To gain an insight into the possible role of CRBP (II) in chicken small intestine, the CRBP(II) contents in cytosols of small intestine of embryonic and post-hatch chicks were determined by enzyme-linked immunosorbent assay. The amount of CRBP(II) per unit DNA in small intestine was low at 15- and 17-day embryonic stage, but rapidly increased around the period of hatching. The increased level was still maintained in 6-week-old chicks, which accounted for 0.9% of total proteins in duodenum. The developmental pattern and the presence of abundant amount of CRBP(II) in chicken small intestine supports the hypothesis that CRBP(II) might play some role in the intestinal absorption of retinoids. Thus the involvement of a tissue-specific cellular retinol-binding protein in the intestinal absorption of retinoids appears to be common in mammalian and avian species.

Key Words cellular retinol-binding protein (type II), development, chicken intestine, purification, enzyme-linked immunosorbent assay

Cellular retinol-binding proteins (CRBP) have been detected in many tissues of all mammalian species thus far examined (1). The presence of CRBP was also reported in chicken (2) and in fish (3). Recently, a second form of CRBP was purified from rat fetuses and it was called CRBP(II) (4). Further investigations revealed that CRBP(II) is abundant in the small intestine of rats (4, 5) and human subjects (6).
suggesting that CRBP(II) might play some role in the absorption of retinoids. However, it was not clear whether the presence of CRBP(II) is widespread in both mammalian species and non-mammalian species. In the present study, we have purified a cellular retinol-binding protein (CRBP(II)) from the cytosol fraction of chicken intestine, and investigated the developmental changes in the content of CRBP(II) in chicken small intestine.

MATERIALS AND METHODS

**Preparation of tissue extracts.** The small intestine (300 g) of three-month-old white leghorn chickens were brought into the laboratory from a slaughter house. Luminal content of the small intestine was extensively rinsed with cold 0.9% NaCl solution and the tissue stored at -80°C until use. The purification procedures were essentially according to those described by Ong (4). The small intestine was cut into pieces with a pair of scissors and homogenized in 2 volumes (v/w) of 0.01 M Tris/HCl, pH 7.5, containing 0.8 mM phenylmethyl sulfonylfluoride (PMSF) in a Waring blender for 60 s. This and subsequent steps were carried out at 0–4°C. Debris was removed by centrifugation at 20,000 × g for 15 min. The supernatant was adjusted to pH 5.0 with glacial acetic acid and centrifuged at 20,000 × g for 15 min to remove precipitated material. The supernatant was adjusted to pH 7.0 with NaOH solution and concentrated to 79 ml by ultrafiltration (YM-5 membrane, Amicon Corp.).

**Column chromatography leading to separation of cellular retinol-binding proteins.** The concentrated sample was submitted to gel filtration on a column (5 × 95 cm) of Sephadex G-75, equilibrated with 0.2 M NaCl, 0.05 M Tris/HCl, pH 7.5. Immediately prior to gel filtration, all-trans-retinol (Sigma) was added, 1 μmol in 0.1 ml of isopropanol. The elution position of bound retinol was determined by measuring the fluorescence of the fractions (excitation at 350 nm, emission at 480 nm). A peak of fluorescence was observed centered at an elution volume of about 1,300 ml. Fractions showing this fluorescence were combined and lyophilized. The lyophilized material was dissolved in 20 ml of distilled water, dialyzed against 0.02 M imidazole/acetate, pH 6.4, and applied to a column (2.6 × 20 cm) of DEAE-cellulose (DE-52, Whatman) equilibrated with the same buffer. The column was eluted with this buffer until the fluorescence and the absorbance at 280 nm of eluate was equivalent to that of the starting buffer. A fluorescence peak was eluted before the gradient elution started (Fig. 1). The column was then eluted with a linear gradient of the imidazole/acetate buffer, from 0.02 M to 0.14 M (total volume 400 ml). A peak with retinol fluorescence and absorbance at 348 nm was eluted in fractions centered at 265 ml of the 400 ml gradient (Fig. 1).

**Purification of CRBP(II)** Material from the first major peak of retinol fluorescence was combined and submitted to the following sequence of steps (see Table 1). The material was submitted first to chromatography on DEAE-cellulose (DE-52, 2.6 × 17 cm) and eluted by a linear 400 ml gradient from 0.01 M to 0.2 M Tris/acetate, pH 8.3. The binding protein was detected in this and subsequent steps by J. Nutr. Sci. Vitaminol.
monitoring the retinol fluorescence, the absorbance of bound retinol at 348 nm, and the absorbance of protein at 280 nm. The fractions from a single peak of retinol fluorescence were combined, concentrated by ultrafiltration. This material was submitted to the second chromatography on CM-cellulose (CM-52, 2.6 x 17 cm) equilibrated with 0.1 M imidazole/acetate, pH 6.2, and eluted by a linear 200 ml gradient from 0.01 M to 0.2 M imidazole/acetate, pH 6.2. The cellular retinol-binding protein was reapplied to the same column, now equilibrated with 0.01 M sodium acetate, pH 5.0. The column was eluted with a linear 500 ml gradient from 0.01 M to 0.2 M sodium acetate, pH 5.0. The cellular retinol-binding protein was submitted to another chromatography on CM-cellulose (1.5 x 18 cm) at pH 6.2, 0.01 M imidazole/acetate with elution by a linear 500 ml gradient from 0.01 M to 0.2 M imidazole/acetate, pH 6.2. The material was then submitted to gel filtration on Sephadex G-50 (2.6 x 90 cm) equilibrated with 0.05 M Tris/HCl, pH 7.5. A single symmetrical peak of retinol fluorescence and absorbance at 348 nm and at 280 nm was obtained. Finally, the fractions containing the cellular retinol-binding protein were combined, mixed with 2 µmol retinol, and submitted to chromatography on DEAE-cellulose (DE-52, 1.5 x 20 cm) at pH 7.2, 0.01 M imidazole/acetate. After all materials showing the retinol fluorescence and absorbance at 280 nm were eluted by the starting buffer (0.01 M imidazole/acetate, pH 7.2), the column was eluted with a linear 400 ml gradient of the imidazole/acetate buffer from 0.01 M to 0.08 M (Fig. 2). Two peaks were observed: one eluted by the starting buffer and the other eluted at 0.03 M imidazole/acetate. Fractions in the two peak regions were separately pooled and concentrated by ultrafiltration.

Preparation of antiserum. Pure CRBP(II), 200 µg, either form A or form B (see blow), was mixed with 1 ml of Freund's complete adjuvant and injected into a New Zealand white rabbit (Japan SLC, Inc., Hamamatsu) according to the procedure described by Ong (4). The mixture was injected subcutaneously on the back (1.7 ml) and intramuscularly into the thigh (0.3 ml). The injection was repeated with Freund's incomplete adjuvant 2, 6, and 8 weeks after the first immunization. One week after the last injection, blood was collected from ear artery. IgG fraction was purified from the antiserum using a Protein A-Sepharose 4B column according to the procedure of Ey et al. (7). The IgG preparation (2 mg) was biotinylated using N-hydroxysuccinimide biotin (120 µg in DMSO) in 0.1 M NaHCO₃ solution for 4 h at room temperature, followed by extensive dialysis against phosphate-buffered saline.

Polyacrylamide gel electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (8) using 15% acrylamide, 1-mm-thick slab gels. Gels were either stained with Coomassie brilliant blue, and when appropriate with silver staining reagent as described by Oakley et al. (9), or the proteins in the gel were electrophoretically transferred to nitrocellulose membrane (5 V/cm, 2 h) and immunoblotting was performed by the method of Tsang et al. (10). The following molecular weight standards (Sigma) were used: bovine serum albumin (66,000), ovalbumin (45,000),...
glyceraldehyde-3-dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100), and alpha-lactalbumin (14,200).

Preparation of intestinal samples of developing chicks. Fertile eggs of the white leghorn breed were obtained from a local hatchery and incubated at 38°C with 60% relative humidity. Small intestines of the embryonic and post-hatch chicks were obtained 15, 17, 19, and 20 days after the start of incubation, and on the day of hatching and 1, 3, 5, 7 days, and 6 weeks after the hatching. Duodenum and jejunileum were separated. The jejunileum of chicks at the age of 1 day or older was further divided into two equal lengths; these segments were referred to as jejunum and ileum. The intestinal segment was flushed with cold 0.9% NaCl solution, cut along its length, and blotted with a wet paper. The tissue was stored at −80°C. The tissue was homogenized with 20 volumes of phosphate-buffered saline containing 0.5 mM PMSF. An aliquot of the homogenate was used for the determinations of protein, DNA, and disaccharidase activities. The homogenate was centrifuged at 105,000 × g at 4°C for 60 min, and the supernatant was used for the determination of CRBP(II).

Assay of CRBP(II). CRBP(II) was quantified by enzyme-linked immunoassay (ELISA) with a slight modification of the procedure described previously for determination of immunoreactive sucrase-isomaltase (11). Briefly, the sample was applied to wells of a microtiter plate (A/2; Costar, Cambridge, Mass., U.S.A.) that had been precoated with IgG of anti-CRBP(II) serum. Immobilized CRBP(II) was then detected by sequential incubation of biotinylated anti-CRBP(II) IgG, avidin-conjugated peroxidase (Vector Laboratories, Inc., Burlingame, Calif. U.S.A.), and 2,2′-azino-bis(3-ethylbenz-thiazoline sulfonic acid) diammonium salt (Sigma). Purified CRBP (II) form A was used as standard.

Assays of disaccharidases. Sucrase, maltase, and trehalase activities were assayed as described by Dahlqvist (12), with 28 mM sucrose, maltose, and trehalose as substrates, respectively.

Protein and DNA determinations. Protein was determined by the method of Lowry et al. (13), with bovine serum albumin as standard. DNA was determined according to Burton (14), with calf thymus DNA as standard.

Absorbance and fluorescence spectra. Absorbance spectra were obtained using a Shimadzu spectrophotometer (UV-265FS). Fluorescence measurements and spectra were determined with a spectrophotofluorometer (Hitachi 650–10S).

RESULTS

Purification of CRBP(II)

The purification sequence for the CRBP(II) is summarized in Table 1. Starting with 300 g of chicken small intestine, we obtained 1.3 mg of pure CRBP(II). Material in intestinal cytosol fraction was first separated by gel filtration chromatography on Sephadex G-75. A symmetrical peak of retinol fluorescence was obtained in the fractions expected for CRBP and CRBP(II) (apparent molecular weight of
Table 1. Purification of CRBP(II) from chicken small intestine.

| Step                        | Recovery of protein<sup>a</sup> (mg) |
|-----------------------------|-------------------------------------|
| After acid precipitation    | 2,844                               |
| Sephadex G-75               | 458                                 |
| DEAE-cellulose, pH 6.4      | 208                                 |
| DEAE-cellulose, pH 8.3      | 29.6                                |
| CM-cellulose, pH 6.2        | 14.4                                |
| CM-cellulose, pH 5.0        | 8.1                                 |
| CM-cellulose, pH 6.2        | 3.0                                 |
| Sephadex G-50               | 1.9                                 |
| DEAE-cellulose, pH 7.2<sup>b</sup> | form A 0.58   |
|                             | form B 0.76                         |

<sup>a</sup>The amount of protein recovered after each step was determined by the method of Lowry et al. (13). <sup>b</sup>CRBP(II) was separated into two forms (A and B) by this step.

14,000–17,000). This material was separated into two forms by ion-exchange column chromatography on DEAE-cellulose at pH 6.4 (Fig. 1). The major portion of the cellular retinol-binding proteins obtained in the void volume was further purified to near homogeneity by subsequent column chromatographies on DEAE-cellulose at pH 8.3, CM-cellulose at pH 6.2, at pH 5.0, and again at pH 6.2, and Sephadex G-50 (Table 1). SDS-PAGE of this preparation revealed the presence of slight amounts of contaminants detected by silver staining. The preparation was thus further submitted to ion-exchange column chromatography on DEAE-cellulose at pH 7.2. Two forms of cellular retinol-binding proteins were separated by this step (Fig. 2). The two forms will be referred to as A and B. Form A was eluted by the starting buffer, though remarkably retarded. Form B was eluted at 0.03 M imidazole/acetate concentration in the present condition. The ratios of absorbance at 348 nm to absorbance at 280 nm of these two peak fractions were 1.1 in form A and 1.0 in form B throughout the peaks.

Gel electrophoretic analysis

The apparent molecular weight of these two forms of cellular retinol-binding proteins was estimated by SDS-PAGE. The apparent molecular weight of form A was 17,200, identical to that of form B (Fig. 3A). The migration of the two forms of the cellular retinol-binding proteins (A and B) was compared with another cellular retinol-binding protein, which was obtained in the earlier column chromatography step on DEAE-cellulose, pH 6.4 (see Fig. 1) and further partially isolated by column chromatography on DEAE-cellulose at pH 8.3. SDS-PAGE of this preparation showed a major band corresponding to a molecular weight of 15,000 with the minor two bands corresponding to molecular weight of 14,600 and 15,400. None of these protein bands was identical to the cellular retinol-binding protein forms.
Fig. 1. Separation of two retinol-binding proteins by chromatography on DEAE-cellulose at pH 6.4. The pooled fractions obtained from the Sephadex G-75 step (see Table 1) were concentrated and dialyzed against 0.02 M imidazole/acetate, pH 6.4, (starting buffer) prior to separation on a DEAE-cellulose column equilibrated with the same buffer. The column was eluted with starting buffer until fraction 82 (5 ml/fraction) when a linear gradient from 0.02 M to 0.14 M imidazole/acetate, pH 6.4, was applied (gradient volume 400 ml). Each fraction was monitored for retinol fluorescence (●, excitation at 350 nm, emission at 480 nm) and absorbance at 280 nm (△) and 348 nm (○). The major fluorescence peak centered at fraction 20 corresponded to CRBP(II) and the minor fluorescence peak centered at fraction 135 corresponded to CRBP. They were pooled separately and subjected to further purification.

A and B (Fig. 3A). When the same gel was further stained with silver, the profiles showed only a single protein in either form A or form B, indicating the purity of the preparations. The antiserum was produced using the mixture of forms A and B. Immunoblotting of duodenum cytosol proteins of chick embryos and developing chicks showed the presence of a single immunoreactive protein throughout the developmental stage examined (Fig. 3B). The immunoreactive protein was not detectable in 15-day, but it was detected in 19-day embryonic duodenum; greater amounts were seen in 7-day and 6-week chick duodena (Fig. 3B). These results also suggested the monospecificity of the antiserum.

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Fig. 2. Separation of two forms of CRBP(II) by chromatography on DEAE-cellulose at pH 7.2. The column was developed with starting buffer (0.01 M imidazole/acetate, pH 7.2) until fraction 36 (5 ml/fraction) when a linear gradient from 0.01 M to 0.08 M imidazole/acetate, pH 7.2, was applied. The concentration of imidazole acetate (dotted line) was monitored by conductivity of the eluates. The same arrangement as in Fig. 1. The fractions indicated by arrows were pooled separately. The two forms are referred to as A and B.

Absorbance and fluorescence spectra of CRBP(II)

The absorbance spectrum of form A is shown in Fig. 4. The absorbance spectrum of form B was identical to that of form A. The spectrum had two major absorbance peaks: the first one at 280 nm and the second one at 348 nm with distinct peaks at 333 nm and at 363 nm (Fig. 4).

The fluorescence excitation and emission spectra of retinol bound to the cellular retinol-binding protein is shown in Fig. 5. The spectra were identical when retinol was bound to either form. The main excitation peak was at 348 nm. In addition, a small peak was observed at 290 nm. The emission spectra displayed a peak at 475 nm.

Developmental changes of CRBP(II) content in chick intestine

Using the ELISA assay system, it was possible to quantify as low as 1 ng/ml of CRBP(II) (Fig. 6). As shown in Fig. 7, CRBP(II) level in 15-day and 17-day embryonic small intestine was very low (less than 1 µg/mg DNA). The amount of...
Fig. 3. Gel profiles of the purified retinol-binding proteins. (A) Protein samples were treated with 2% sodium dodecyl sulfate, 5% mercaptoethanol in boiling water for 2 min, and analyzed in a 1-mm-thick 15% polyacrylamide slab gel according to the method of Laemmli (8). The gel was stained with Coomassie brilliant blue. Lane 1, molecular weight standards; lane 2, CRBP(II) form A, 1 µg; lane 3, CRBP(II) form B, 1 µg; lane 4, mixture of CRBR(II) forms A and B, 0.5 µg each; lane 5, partially purified CRBP (see Fig. 1), 1 µg; lane 6, mixture of CRBP(II) forms A and B and CRBP, 0.5 µg each. (B) Immunodetection of CRBP(II). Proteins of cytosol fraction of duodenal homogenate (5%, w/v) from 15-day (lane 1) and 19-day (lane 2) chick embryo and 7-day-old (lane 3) and 6-week-old chicks (lane 4) were separated by SDS-PAGE, transferred to nitrocellulose membrane and detected with anti-CRBP(II) antibody. Bound antibody was stained with peroxidase coupled to anti-rabbit IgG.

CRBP(II) slightly increased until the 20-day embryonic stage when the level was 5.85 ± 1.31 (µg/mg DNA, mean ± SEM) in duodenum and 6.19 ± 1.20 (µg/mg DNA) in jejunum. During the subsequent 48 h, i.e., by day 1 after hatching, the levels of CRBP(II) increased 18 times in duodenum and 9 times in jejunum. Thereafter the CRBP(II) contents per DNA did not change considerably. In 6-week chicks, slightly higher values of CRBP(II) content were obtained in duodenum (207 ± 32 µg/mg DNA) and in jejunum (125 ± 25 µg/mg DNA). On the other hand, the CRBP(II) content in ileum diminished to a low level (8.1 ± 0.5 µg/mg DNA) (Fig. 7). The amount of CRBP(II) in 6-week chick duodena was abundant, amounting to 0.9% (w/w) of the total duodenal proteins.

The changes in the levels of CRBP(II) during the perinatal development of the intestine was compared with that of microvillar disaccharidases. As shown in Fig. 8, the developmental patterns of sucrase, maltase, and trehalase activities in chick duodena were similar to that of CRBP(II), except for the time of spurt; the greatest
increase of disaccharidase activities was seen between day one and day three after hatching (Fig. 8), whereas the rapid increase of CRBP(II) occurred around the time of hatching (Fig. 7).

DISCUSSION

We have identified and purified CRBP(II) from chicken small intestine. During the course of purification, two molecular forms of cellular retinol-binding protein were identified; the predominant one with a greater apparent molecular weight (17,200) and the other cellular retinol-binding protein with a smaller molecular weight (15,000). The predominant form of the cellular retinol-binding proteins eluted in the starting buffer on DEAE-cellulose at pH 6.4, which was previously reported as rat CRBP(II) (4). The absorbance spectrum and the fluorescence spectra of this predominant cellular retinol-binding protein were typical for the cellular retinol-binding proteins (4). These results suggest that the cellular retinol-binding protein purified in the present study is CRBP(II) of chicken intestine. The characteristics of the minor form of cellular retinol-binding protein thus far examined, i.e., the apparent molecular weight and the elution profile on DEAE-cellulose column chromatography, were similar to those of rat CRBP (4),
Fig. 6. Enzyme-linked immunosorbent assay for CRBP(II). Increasing amounts of CRBP(II) form A were applied to wells of microtiter plates, which were precoated with protein A-purified anti-CRBP(II) IgG. Biotinylated anti-CRBP(II) IgG was then added and the bound biotinylated IgG was detected by avidin-peroxidase conjugate, followed by color development with 2,2-azino-bis(3-ethylbenz-thiazoline sulfonic acid) diammonium salt. The abscissa indicates the log values for the amounts of CRBP(II).

Fig. 7. Perinatal development of CRBP(II) in chick small intestine. The content of CRBP(II) was determined by enzyme-linked immunosorbent assay as described in "MATERIALS AND METHODS." H, hatching; ●, duodenum; ▲, jejunum; ×, ileum. Each value represents mean ± SEM of 3 chicks. The jejunileum of embryonic and 0-day-old chicks was not separated into jejunum and ileum, and the values for jejunoileum are shown.

suggesting that this minor form represents chicken CRBP. Although the purified CRBP(II) was separated into equal amounts of two forms by DEAE-cellulose, at pH 7.2 (Fig. 2), the properties of these two forms (A and B) thus far examined were identical, i.e., apparent molecular weight was the same, and fluorescence spectra and absorbance spectra were identical. Thus, it appears that the two forms of CRBP(II) originated from the same peptide. A separation of CRBP(II) into two forms by ion-exchange column chromatography was also reported in rat CRBP(II) (4). The present study supports the existence of intestinal form of cellular retinol-binding protein (type II), which is distinct from the commonly known form of CRBP (15). The CRBP(II) has been found in cytosol from two species, i.e., rat and human. Chicken is now to be added to the list.

The physiological significance of abundance of CRBP(II) remains obscure. A
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Fig. 8. Perinatal development of disaccharidase activities in chick duodenum. —○—, maltase activity; —△—, sucrase activity; —□—, trehalase activity. The same arrangement as in Fig. 7. Maltase activity and sucrase activity in 6-week-old chicks were 723±70 and 152±9 (µmol/mg DNA/h), respectively.

Histochemical evidence that the localization of CRBP(II) was restricted to the villus cells of small intestine in rats (5) and in chicken (Goda and Takase, unpublished results) strongly suggested that CRBP(II) might play some role in absorption of retinol. Furthermore, it has been reported using microsomes of rat small intestine that the retinol bound to CRBP(II) was esterified more efficiently than the retinol administrated as a complex with CRBP or serum retinol-binding protein (16). Recently, MacDonald and Ong (17) demonstrated that rat CRBP(II) bound trans-retinaldehyde as well as trans-retinol, whereas rat CRBP did not bind trans-retinaldehyde. The chicken CRBP(II) prepared in the present study also bound trans-retinaldehyde as evidenced by the results that the bound retinol was displaced by 10 times greater amount of trans-retinaldehyde; it was not displaced by retinoic acid or palmitic acid (Goda and Takase, unpublished results). Thus it appears that the endogenous ligands of CRBP(II) are not only retinol but also retinaldehyde which might be derived from β-carotene.

Using a monospecific antiserum prepared against chicken CRBP(II), we have developed a sandwich-type ELISA of chicken CRBP(II). No immunoreactive materials were detected in 6-week-old chicken serum, and the crude chicken CRBP preparation did not react with this antiserum (Goda and Takase, unpublished results), suggesting that the presence of CRBP and/or serum retinol binding protein in the tissue preparations did not affect the results of CRBP(II) determinations. We have demonstrated in the present study that in chicken CRBP(II) exhibits a developmental pattern similar to disaccharidases, which are known to play a crucial role in the absorption of disaccharides.
role in digestion and absorption of carbohydrates and have been previously characterized (18, 19); both increased rapidly around the period of hatching. The difference in the time of spurt between CRBP(II) and disaccharidases might suggest that the induction of CRBP(II) and disaccharidases is triggered by different humoral and/or dietary factor(s). These factors should be elucidated in future studies. We speculate that chicken CRBP (II) might be involved in the absorption and esterification of retinoids in small intestine. The presence of abundant amount of CRBP(II) in chicken small intestine suggests that the involvement of a tissue-specific cellular retinol-binding protein in the intestinal absorption of retinoids might be common between mammals and birds.

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