Gene Expression of Cellular Retinol-Binding Protein I (CRBP I) is Affected by Dietary Proteins in the Rat Liver

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Summary The effect of dietary proteins and vitamin A status on the gene expression of cellular retinol-binding protein I (CRBP I) was studied in the rat liver. The gene expression was estimated as amounts of transcript (mRNA) by Northern blot analysis using rat CRBP I cDNA. Though vitamin A status is known to positively regulate the gene expression of CRBP I in the extrahepatic tissues, in the present study we observed that the amount of the CRBP I transcript in liver was neither reduced by vitamin A-deficiency, nor affected by replenishment with an excess dose of all-trans retinoic acid. These results indicate that in the liver, different from the extrahepatic tissues, the gene expression of CRBP I may not be controlled by vitamin A. However, when the rats were fed on the diets that differed in dietary proteins, the gene expression of CRBP I in liver was enhanced by higher quality and quantity of dietary proteins, though no effect of dietary proteins was observed upon the hepatic contents of retinol. The concentrations of serum retinol were almost proportional to the mRNA levels of CRBP I. In contrast, the hepatic gene expression of another retinol-binding protein, RBP, and one subtype of retinoic acid receptor, RARα was not influenced in the nutritional condition tested here. Our findings suggest that the gene expression of CRBP I in liver may be under control of the intake of dietary proteins. Thus, it is likely that in the light of the function of CRBP I on cellular transport and metabolism of retinol, dietary proteins may affect the actions of vitamin A in the extrahepatic tissues through changing the amounts of CRBP I in liver.

Key Words vitamin A, gene expression, cellular retinol-binding protein I (CRBP I), dietary proteins, rat liver

It is well documented that vitamin A (retinol and its derivatives) are essential...
for a wide range of functions such as vision, reproduction, growth and cellular differentiation in animals (1). Moreover, studies have shown that a putative active compound of vitamin A, retinoic acid, is involved in vertebrate development and embryogenesis, probably as a natural morphogen (2). Recent discovery of nuclear retinoid receptors has provided understanding of the underlying mechanisms of retinoid signal transduction based on the gene expression (3, 4). Two classes of nuclear retinoid receptors (three subtypes of each of RAR and RXR) have been characterized so far, and shown to act as a ligand-dependent transcription factor like steroid/thyroid hormone nuclear receptors (3–5). Moreover, from the analysis of naturally occurring response elements, consensus retinoic acid response elements (target enhancers for the retinoid receptors) have been identified (6). Although the framework of retinoid signal transduction based on receptor-mediated gene expression has been revealed, it is still unclear how vitamin A in the cytosol is transported into the nucleus (6, 7).

After absorbing pro-vitamin A in the intestine, retinol converted from pro-vitamin A is esterified as retinyl esters, then transported to the liver, a major storage organ for vitamin A. Responding to the demand of extrahepatic tissues, retinyl esters are hydrolyzed and the resultant retinol is bound for serum retinol-binding protein (RBP) in liver. The complex of retinol with RBP is secreted in a strictly regulated manner and circulated as a heterocomplex with transthyretin (TTR). Target tissues uptake retinol from this heterocomplex, probably through its specific cell-membrane receptor (8). The incorporated retinol is oxidized via retinal into retinoic acid as the ligand for the nuclear retinoid receptors. During these processes in the cytosol, many retinoid binding proteins such as cellular retinol-binding proteins (CRBP I and II) and cellular retinoic acid-binding proteins (CRABP I and II) are associated or/and involved as substrates for retinoid metabolism and its transport (9). Considering their functions, it is speculated that the amounts of retinoid binding proteins profoundly affect the retinoid signaling through modulating retinoid transport (9). Such idea is indeed supported by the facts that the ligand-mediated regulation of the gene expression is observed among many retinoid binding proteins including the nuclear receptors (10–14).

As apo- and holo-forms of CRBP I function as substrates for hydrolysis of retinyl esters to produce retinol and retinol conversion into retinal, respectively (9, 15), the metabolism of retinoid in a given tissue expressing CRBP I may be affected by the amounts of CRBP I. In the previous observations in our laboratory, a mutual relationship between vitamin A and protein metabolism is speculated from the animal experiments where the dietary proteins affected vitamin A metabolism (16). Therefore, the present study was undertaken to investigate whether the dietary proteins as well as vitamin A status would affect the gene expression of CRBP I in rat liver. We present here an evidence that the CRBP I gene expression in liver is affected by dietary protein conditions, whereas no influence of vitamin A status was seen. Under these nutritional conditions the mRNA levels of another retinol-binding protein, RBP, and one subtype of retinoic acid receptors, RARz,
were unchanged. Thus, the present study indicates that the hepatic expression of CRBP I gene may be affected by dietary proteins.

MATERIALS AND METHODS

Preparation of cDNAs of the rat CRBP I and rat RBP genes. The cDNA of rat CRBP I gene was prepared by RT (reverse transcription)-PCR (polymerase chain reaction). Poly(A)⁺ RNA was prepared by oligo(dT)-cellulose chromatography from the rat liver (17). Total cDNAs were synthesized with a poly(dT) primer (18 mer) using AMT reverse transcriptase (Life Science, Inc.) in 20 μl. Using 1 μl of this solution, the full-length rat CRBP I cDNA (18) was generated with a pair of oligonucleotides (nucleotide+1 to +18 and +385 to +402 of the CRBP I DNA sequence). The rat RBP cDNA (19) was obtained in the same way with a pair of oligonucleotides (nucleotide+1 to +18 and +513 to +530 of the rat RBP sequence). The sequences of the cDNAs were verified by dideoxynucleotide sequencing (20).

Animals and diets. Male weanling rats of the Wistar strain (3 weeks old) were used throughout the experiments. As reported previously (11), the retinol-deficient rats (four rats) were prepared by feeding on a vitamin A-deficient diet (20C-A in Table 1), while the control rats (four rats) were fed on a vitamin A supplemented diet (20C). Serum and liver contents of total retinol were determined by HPLC with authentic retinol as the external standard as previously described (11). Animals were judged as being in a retinol-deficient state when serum retinol levels were below 1.8 μg/dl (normal levels are around 40 μg/dl) and hepatic contents of retinol were depleted to 2.5 μg/g of liver (normal levels are about 30 μg/g of liver) (11). On the day 35, the vitamin A-deficient rats were sacrificed and samples were prepared. The replenished rats (four rats) were made by intragastrical administration of all-trans retinoic acid (100 μg in 0.2 ml of soybean oil) to the vitamin A-deficient rats, and 4 h later the rats were subjected for analyses.

The composition of the diets used for the study of dietary proteins are shown in Table 1. To study the direct effect of dietary protein on the gene expression, eliminating the influence of endogenous vitamin A, the hepatic retinol in the rats (the initial body weight, 52.3 ± 2.1 g) was tempted to be depleted by feeding on a 5% gluten diet depleted of vitamin A (5G-A) for 1 week. Then, six rats/group were fed on the diets (5G, 5C, and 20C) for 2 weeks under the experimental conditions as previously described (11). Final body weights were 66.7 ± 4.2 g (5G), 72.4 ± 3.6 g (5C), and 154.7 ± 5.3 g (20C), respectively.

RNA isolation and Northern blot analysis (11). Total RNA was isolated from rat tissues by the acid guanidinium thiocyanate/phenol/chloroform method (21). Total RNA (50–100 μg) was electrophoresed through 1% agarose/1.1 M-formaldehyde gels and transferred to nitrocellulose filters (Schleicher and Schuell, BAS 85). The full-length cDNAs of rat CRBP I, mouse RARα and rat RBP were
Table 1. Composition of the diets.

<table>
<thead>
<tr>
<th>Components</th>
<th>20C</th>
<th>5C</th>
<th>5G</th>
<th>20C-A</th>
<th>5G-A (%)</th>
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</thead>
<tbody>
<tr>
<td>Casein (vitamin A free)</td>
<td>20.0</td>
<td>5.0</td>
<td>—</td>
<td>20.0</td>
<td>—</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>—</td>
<td>—</td>
<td>5.0</td>
<td>—</td>
<td>5.0</td>
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<tr>
<td>Wheat starch</td>
<td>65.0</td>
<td>80.0</td>
<td>80.3</td>
<td>65.0</td>
<td>80.3</td>
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<tr>
<td>Cellulose powder</td>
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<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Soybean oil</td>
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<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>DL-Methionine</td>
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<td>0.3</td>
<td>—</td>
<td>0.3</td>
<td>—</td>
</tr>
<tr>
<td>Choline chloride</td>
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<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Mineral mix(^1)</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin mix(^2) (vitamin A free)</td>
<td>1.0(^b)</td>
<td>1.0(^b)</td>
<td>1.0(^b)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^1\) Mineral mix (mg/diet-100 g): CaHPO\(_4\), 1,750; NaCl, 259; K\(_2\)C\(_6\)H\(_5\)O\(_7\)•H\(_2\)O, 770; K\(_2\)SO\(_4\), 182; MgO, 84; MnCO\(_3\), 12.25; FeC\(_6\)H\(_5\)O\(_7\)•H\(_2\)O, 21.0; ZnO, 5.6; CuCO\(_3\), 1.05; KIO\(_3\), 0.035; Na\(_2\)SeO\(_3\)•2H\(_2\)O 0.035; CrK\((SO_4)_2\)•12H\(_2\)O, 1.925.  
\(^2\) Vitamin mix (mg/diet-100 g): thiamin-HCI, 0.6; riboflavin, 0.6; pyridoxine-HCl, 0.7; nicotinic acid, 3.0; Ca-pantothenate, 1.6; folic acid, 0.2; biotin, 0.02; V.B12, 0.001; Menaquinone, 0.005; V.D\(_3\), 0.0025; V.E, 5.0.  
Supplemented with retinylpalmitate (0.22 mg/diet-100 g).

labeled with \[^{32}\text{P}\]dCTP by the random priming method. Hybridization was carried out at 42–45°C for 18 h in 5×SSPE (1×SSPE = 1.15 M NaCl/10 mM Na\(_2\)H\(_2\)PO\(_4\)/1 mM EDTA, pH 7.0), 50% formamide, 0.2 mg of denatured salmon sperm DNA/ml and 1 × Denhardt’s reagent. The most stringent wash was performed at 65°C in 0.1×SSPE containing 1.0% SDS and 0.03% sodium pyrophosphate. Filters were dehybridized by 15 min treatment in 0.1×SSPE/0.1% SDS at 90°C. A 1.5 kb fragment of rat β-actin cDNA was used as a β-actin probe to confirm that the same amounts of RNA were loaded. Autoradiography were usually performed with two different lengths of exposure (3–10 days for CRBP I, 1–6 days for RBP and RAR\(_\alpha\), and 1–2 days for β-actin transcripts) at −80°C with intensifying screens. To obtain quantitative data on CRBP I, RBP, and RAR\(_\alpha\) transcripts we used densitometric analysis of the autoradiograms, with β-actin transcripts as internal control in a given tissue. The relative abundance of CRBP I, RBP, and RAR\(_\alpha\) transcripts is shown as the M±SD for at least four samples from each different rat after normalizing to the β-actin mRNA level.

Statistical analysis. Student’s t-test was applied to determine the significance of differences in mean values between the groups, with \(^*p<0.01\) being considered as significant.

RESULTS

The effect of vitamin A status on the gene expression of cellular retinol-binding protein I (CRBP I) in rat liver

It is well described that vitamin A status up regulates the transcript levels of

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many retinoid binding proteins including nuclear retinoid receptors (10–14). The mRNA level of CRBP I in liver was estimated in the vitamin A-deficient rats and the replenished rats with an excess amount of all-trans retinoic acid (100 μg). For this purpose, the Northern blot analysis was employed. Total RNA extracted from various rat tissues was subjected to a agarose-gel electrophoresis, then on the blotted membrane hybridized with [32P]-labeled rat CRBP I cDNA probe. The cDNAs of rat RBP and mouse RARα for comparison and the rat actin cDNA as an internal control were also used as probes.

Our preliminary results confirmed the differential expression of the CRBP I gene in various tissues, in good agreement with the previous reports (10,12,14). Furthermore, as we expected, the transcript size was about 0.7 kb and in liver the amount of the CRBP I mRNA was abundant (data not shown).

Fig. 1. The effect of vitamin A status on the cellular retinol binding protein I (CRBP I) gene expression in the rat liver. The vitamin A-deficient rats (V. A deficiency) were made as described in MATERIALS AND METHODS. One hundred micrograms of all-trans retinoic acid was given to the retinol-deficient rats (+ RA), and 4h later the tissues of the rats and control rats fed on 20C(C) were subjected to Northern blot analysis. Only the results of Northern blot analysis on the liver from one rat of each group were shown representatively (A). Relative signal intensity of the transcripts was expressed as the average of more than four rats in a given group (B). The hepatic and serum contents of retinol (C) were estimated by HPLC as described in MATERIALS AND METHODS. Values are M±SD (n=4). Significant differences are indicated at *p<0.01 from the control rats (C).
Vitamin A-deficiency caused a clear reduction of the CRBP I mRNA level in lung (data not shown), as reported previously (14). However, in the same rats, no reduction on the CRBP I mRNA level in liver was seen (Fig. 1). Moreover, a replenishment study on the vitamin A-deficient rats showed that, though a given retinoic acid recovered the level of the CRBP I in lung within a short period (4 h) (data not shown), but it elicited no effect on the hepatic mRNA level of CRBP I (Fig. 1). Likewise, the gene expression of RBP and RARα was not affected by vitamin A status. From these observations, we conclude that the gene expression of CRBP I in liver is not affected by vitamin A status.

The gene expression of CRBP I is influenced by dietary proteins

The relationship between the dietary conditions of protein and the CRBP I gene expression was examined, because it was pointed out from our previous findings that the retinol contents in the tissues were affected by the quality and quantity of dietary proteins (16). These observations imply that the dietary

![Diagram](Fig. 2. The effect of dietary proteins on the CRBP I gene expression. The rats were fed on the 5% gluten diet depleted of retinol (5G-A) for 1 week, and then (divided into three groups and fed on the diets that differed in dietary proteins 5% gluten (5G), 5% casein (5C), and 20% casein (20C), see Table 1) for 2 weeks. The Northern blot analysis of the tissues was performed as described in Fig. 1 and the relative abundance of CRBP I, RBP, and RARα transcripts is shown. Serum and hepatic contents of retinol were measured by HPLC. Values are M±SD (n=6). Significant differences are indicated at *p <0.01 from the results in the rats fed on 5G-A.)

proteins may affect the amounts of the retinoid binding proteins, thereby modulating the vitamin A status. In the light of a key function of CRBP I upon the metabolism and transport of retinol, it is feasible that the gene expression of the CRBP I gene expression in liver may be affected by dietary condition.

To clarify these points, the effect of dietary proteins on the CRBP I mRNA level in liver was studied. To eliminate the endogenous retinol and observe the dietary effect on the accumulation of retinol in liver, the rats were fed on a 5% gluten diet depleted of retinol (5G-A) for 1 week. As shown in Fig. 2, the serum and hepatic contents of retinol were indeed lowered by this feeding. Then the rats were divided into three groups, and fed on either a 5% gluten diet supplemented with retinol (5G), a 5% casein diet (5C), or a 20% casein (20C) for 2 weeks. As shown in Fig. 2, even in the rats fed on 5G the hepatic total retinol contents of the rats were recovered up to the same levels, almost as the normal one (11). Under these conditions, the levels of CRBP I transcript in liver were positively regulated by the quality and quantity of dietary proteins. Namely, when compared to the hepatic CRBP I mRNA level in the rats fed on 5G-A for 1 week, a significant increase was observed in the rats fed 5C and 20C, but not in the rats fed on 5G. Thus, these results on the rats fed on 5G suggested again that the hepatic expression of CRBP I gene is not controlled by vitamin A status. As a good internal control, the mRNA levels of RBP and RARα were not affected throughout these experiments. It is noted that serum retinol concentrations were almost proportional to the levels of CRBP I transcript in liver, though the hepatic retinol contents were not changed.

**DISCUSSION**

The intracellular transport of vitamin A into the nucleus is supposed to be strictly regulated in tissue-specific and developmental ways, when considering the pleiotropic actions of vitamin A (1, 2). These transports are undertaken by specific retinoid binding proteins. One of such binding proteins, CRBP I, plays a key role in retinoid metabolism and transport, since holo-CRBP I (retinol-bound CRBP I) serves as a substrate for the conversion of retinol into retinal, and hydrolysis of retinyl esters to produce retinol is enhanced by apo-CRBP I level (9).

In our previous studies using chicks, it was found that the metabolism of vitamin A was modulated by dietary proteins, suggesting a mutual relationship between the vitamin A and protein metabolisms (15). Therefore, in order to clarify this point we chose CRBP I from a view of its importance in vitamin A metabolism, and the present study was undertaken to explore whether the gene expression of CRBP I might be influenced by dietary proteins. We showed here that the level of CRBP I transcript in liver was indeed affected by the dietary proteins, but not by vitamin A status.

Our preliminary experiments confirmed the tissue-specific expression of the CRBP I gene in the rats (data not shown), as previously reported by other groups
Such differential expression of the CRBP I gene in various tissues is supportive for the function of CRBP I for vitamin A metabolism, because its high abundance in liver reflects the function of this organ as major organ on the storage and transport of retinol. Moreover, in good agreement with previous observations (12, 14, 18), we confirmed that the gene expression of CRBP I in lung was clearly affected by vitamin A status. However, notably we also found that the hepatic expression of this gene was not influenced by vitamin A status. We could not rule out a possibility that retinoic acids may not be depleted thoroughly in the liver of the vitamin A-deficient rats to influence the gene expression. Nevertheless a clear difference of response to retinoid status between liver and lung may account for, at least partially, the tissue-specific actions of vitamin A.

When the hepatic retinol contents were almost normal, the quantity and quality of dietary proteins clearly affected the gene expression of CRBP I in liver. The hepatic mRNA levels of CRBP I were potentiated by the higher content and the better quality of proteins. The effect of dietary proteins on the CRBP I transcript level in liver is possibly not due to the altered concentrations of the serum and hepatic retinol, since vitamin A status did not affect the hepatic amount of CRBP I mRNA as shown in Fig. 1. These findings suggest that the dietary proteins may be another factor controlling the gene expression of CRBP I in liver. It should be noted that such regulatory mechanism is specific for CRBP I, since the gene expression of RBP and RARα in liver was not changed by any condition tested here. The dietary proteins, which affect deeply protein metabolism, may modulate also the metabolism of vitamin A through changing the CRBP I levels. As the serum retinol levels were almost proportional to the amounts of the CRBP I mRNA levels in liver with the constant levels of hepatic retinol, we raise a possibility that lower contents of CRBP I (apo-form) in liver may decrease the hydrolysis of retinyl esters to produce free retinol, thereby reducing the secretion of the retinol-bound RBP. In fact, it is well described that the levels of serum RBP are reduced by protein-calorie malnutrition (PCM) (22). Such reduction of serum RBP by PCM may be due to the low intake of dietary proteins, as suggested from the present study. Thus, our previous hypothesis about the correlation of vitamin A metabolism with protein metabolism is strengthened by the present observations.

Recent discovery of nuclear retinoid receptors gave us an insight upon the molecular mechanism of retinoid actions (3–5). As nuclear retinoid receptors have been demonstrated as the member of a superfamily of the steroid/thyroid hormone receptors, which act as a ligand-dependent transcription factor, most of retinoid actions are speculated to be governed by gene regulation mediated with these receptors (23). The functional analysis of the CRBP I gene promoter using the retinoid receptor cDNA has revealed that a retinoic acid response element (RARE) is located in its promoter (6). Our findings on the retinoid-regulated gene expression of CRBP I in the rat lung were supported by these results (10, 18). Taken together with these findings, the failure of CRBP I gene expression to respond to retinoid in liver in vivo clearly indicates the involvement of an additional
tissue-specific transcription factor in the regulatory element of CRBP I gene promoter. Therefore, it is most likely that near the RARE there may be additional regulatory sequence, whose function may be controlled by the dietary proteins. Currently, to test possibility, the functional analysis of the promoter in a transient expression experiment (19), besides of run-on assay from the rats fed on various diets, is under investigation in our laboratory.

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