Effect of Retinol Status on Retinol-binding Protein Biosynthesis Rate and Translatable Messenger RNA Level in Rat Liver*

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Studies were conducted to explore the role of retinol in the control of the rate of synthesis of plasma retinol-binding protein (RBP) in the liver of the rat. Previous studies have shown that nutritional retinol status strongly influences RBP secretion from the liver cell. Both the in vivo relative rate of RBP synthesis and the in vitro translational level of RBP-specific mRNA were examined in normal vitamin A, retinol-depleted, and retinol-repleted rats. The relative rate of RBP synthesis was estimated by measuring the extent of incorporation of [3H]leucine, [3H]lysine, and [3H]phenylalanine after a 12-min pulse label into immunoprecipitable RBP, relative to the incorporation of these amino acids into total liver trichloroacetic acid-precipitable protein. The level of translatable RBP-specific mRNA was quantitated in vitro by translation of rat liver poly(A)+RNA in the rabbit reticulocyte lysate protein-synthesizing system. The amount of newly synthesized RBP was determined relative to the amount of newly synthesized total protein. Both the relative rate of RBP synthesis (approximately 0.26%) and the translatable level of RBP-specific mRNA (approximately 0.14%) were found to be constant regardless of the retinol status of the rats. These results indicate that retinol, the molecule that RBP specifically binds and transports, does not appear to control the rate of synthesis of RBP or the translatable level of RBP-specific mRNA in the liver of the rat. Regulation of plasma RBP levels by retinol must be exercised at a locus beyond that of RBP synthesis.

Retinol-binding protein, the specific plasma transport protein for vitamin A, serves to transport retinol from its storage site in the liver to peripheral target tissues of the body (see Refs. 1 and 2 for recent reviews). This protein is synthesized in the liver initially as a precursor, pre-RBP† (approximately 24,000 daltons mass), which is co-translationally processed by microsomal membranes of the rough endoplasmic reticulum to RBP (20,000 to 21,000 daltons) (3). RBP has one binding site for a single molecule of retinol and is secreted into the plasma as the retinol-RBP complex (holo-RBP) (4, 5). The holo-RBP in the plasma strongly interacts with plasma prealbumin and normally circulates as a 1:1 molar RBP-prealbumin complex.

Vitamin A mobilization from the liver appears to be regulated by factors that control the rates of RBP synthesis and secretion. One factor which specifically regulates RBP secretion from the liver is the nutritional retinol status of the animal. Studies in the rat have shown that in the retinol-depleted state, the secretion of RBP from the liver is blocked, resulting in the accumulation of an enlarged pool of apo-RBP in the liver and a concomitant decline in serum RBP levels (6, 7). Conversely, repletion of retinol-deficient rats with retinol stimulates the rapid secretion of RBP from the expanded liver pool into the plasma (7). Similar effects of retinol on RBP secretion have been observed with differentiated hepatoma cells in culture in vitro (8).

Other studies on RBP metabolism in the liver have shown that the drug cholecalciferol strongly inhibits RBP secretion, suggesting that microtubules may be involved in this process (9). In addition, the production of RBP by differentiated hepatoma cells in culture has been shown to be stimulated by glucocorticoid hormones and, particularly, by the corticoid analog, dexamethasone (10).

We now report evidence that the rate of RBP biosynthesis in liver is not influenced by changes in the retinol status of the animal. Retinol thus appears to regulate RBP secretion rate without affecting RBP synthesis.

MATERIALS AND METHODS

Animals—Twelve male weanling rats were obtained from the Holtzman Co., Madison, WI. All rats were housed individually in an air-conditioned room with a mean temperature of 22 °C and had free access to both food and water. Upon arrival, the rats were randomly divided into three groups of four rats each (called normal vitamin A, retinol-depleted and retinol-repleted) (3). RBP was one binding site for a single molecule of retinol and is secreted into the plasma as the retinol-RBP complex (holo-RBP) (4, 5). The holo-RBP in the plasma strongly interacts with plasma prealbumin and normally circulates as a 1:1 molar RBP-prealbumin complex.

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at the time of the experiment were not significantly different from each other, with the mean body weight ± S.E. being 370 ± 12, 347 ± 10, and 357 ± 24 g for the normal, retinol-depleted, and retinol-repleted rats, respectively.

**Relative Rate of RBP Synthesis—** Each rat was injected intraperitoneally with 500 μCi/100 g body weight of a mixture containing equimolar amounts of radioactivity from [3H]leucine, [3H]lysine, and [3H]phenylalanine (New England Nuclear, specific activities 110 Ci/mmol, 70 Ci/mmol, and 100 Ci/mmol, respectively). These amino acids were chosen because of their relative abundance in RBP (5). Twelve min after injection, before the newly synthesized secretory proteins (12) (e.g., albumin and prealbumin) were secreted into the plasma, the rat was decapitated and the serum and liver collected. Four g of liver were vigorously homogenized in 3 volumes of 10 mM Na phosphate buffer, pH 7.4, containing 125 mM NaCl, 1% Triton X-100, 1% Na deoxycholate, and 10^-4 M phenylmethylsulfonyl fluoride. This homogenate was then centrifuged at 100,000 x g at 2 h. RBP in liver is mainly found in association with the microsomal fraction (11). We have found that the use of 1% Triton X-100 and 1% Na deoxycholate in the homogenizing solution results in the solubilization of greater than 97% of the immunoreactive liver RBP, with the recovery of virtually all of the immunoreactivity in the supernatant of the liver homogenate. Repetition of the microsomal pellet with the homogenizing buffer resulted in the detection by radioimmunooassay of only trace amounts of RBP.

Radioactivity present in total liver protein was determined on the whole liver homogenate by trichloroacetic acid precipitation on filter paper discs as described by Mans and Novelli (16). For each rat, [3H] RBP was immunoprecipitated from the liver homogenate after centrifugation of the homogenate for 2 h at 100,000 x g. Samples of the resulting liver homogenate supernatant were immunoprecipitated four separate times utilizing a specific rabbit anti-rat RBP antiserum (5) in the same manner as previously described (3). Immunoprecipitated products were subjected to SDS and 12.5% polyacrylamide slab gel electrophoresis (15). To quantitate the radioactivity in RBP, each lane of the gel was cut into 2-mm slices and the slices were solubilized in 0.5 ml of 30% H2O2 at 70 °C. After solubilization of the gel slices, 10 ml of Hydrofluor (National Diagnost Inc.) were added and the samples were assayed for [3H] in a Packard liquid scintillation counter, model 3255. Samples containing the immunoprecipitated [3H]RBP were counted until a sufficient number of counts were recorded to ensure less than a 1% counting error. The molecular weight of the immunoprecipitated RBP, estimated from its electrophoretic mobility relative to that of known standards (3), was compared with that of purified rat serum RBP isolated in the same manner as previously reported (16).

In order to determine the efficiency of recovery of [3H]RBP from each liver homogenate supernatant, a trace amount of [35S]RBP was added to each 100,000 x g rat liver homogenate supernatant. The RBP was immunoprecipitated in the exact manner as above and the efficiency of recovery of the [35S]RBP calculated. The efficiency of RBP immunoprecipitation between animals within a treatment and among the three treatments was very similar. The observed average recovery of [35S]RBP was 55%. All values listed in the tables and under "Results" represent values that have been corrected to account for this efficiency of RBP recovery.

**Translatable Level of RBP-specific mRNA—** Total RNA was isolated from 2-g liver samples, obtained immediately after killing the same rats utilized in the experiment described above on the relative rate of RBP synthesis, by the guanidine HCI method of Chirgwin et al. (17). RNA enriched in poly(A)+ RNA was obtained by oligo (dT)-cellulose affinity chromatography as described by Pickett et al. (18). RNA was quantitated utilizing the extinction coefficient of 1 A260 unit for 50 μg of RNA. All poly(A)+RNA samples had an absorbance ratio (260/280 nm) of approximately 2.0.

The poly(A)+RNA was analyzed in the micrococcal nuclease-treated rat reticulocyte in vitro protein-synthesizing system (19) as described previously (3). Each reaction mixture contained 19 amino acids and [35S]methionine. Four separate translation assays were carried out per rat. Newly synthesized [35S]methionine-labeled RBP was immunoprecipitated and quantitated; the efficiency of recovery of added [35S]RBP was calculated; and total [35S]methionine-containing trichloroacetic acid-precipitable protein was determined, all in the same manner as described above. The efficiency of RBP recovery from the translation mixture was averaged 57% and was very similar regardless of the source of the poly(A)+RNA. All values presented under "Results" have been corrected to account for this efficiency of RBP recovery.

**Vitamin A and RBP Assays—** RBP in the liver homogenate 100,000 x g supernatant and in the serum was quantitated by a specific and sensitive radioimmunooassay as described before (6, 10, 11). Vitamin A in liver and serum was determined by the fluorometric analysis method of Thompson et al. (20) using the precautions described by Muto et al. (6). The protein concentration of liver was determined by the method of Bradford (21) using a mixture of human albumin and human globulin (Sigma Chemical Co.) as a standard.

**Statistical Analyses—** All group data are reported as mean ± the standard error of the mean. Statistical comparisons were made by one-way analysis of variance (22), and when significant differences were detected, the appropriate comparisons were made by Tukey's w-procedure.

**RESULTS**

**Vitamin A and RBP Levels—** As a prerequisite for these studies, it was necessary that the rats fed the chosen diets demonstrate the established biochemical parameters of the normal, retinol-depleted, and retinol-repleted states. Table I lists the levels of RBP and vitamin A, in both the liver and serum, in the three experimental groups of animals. Both the vitamin A and RBP levels in serum and liver exhibited the same vitamin A-related pattern previously reported (6, 7).

**Relative Rate of RBP Synthesis—** The incorporation of [3H]-labeled leucine, lysine, and phenylalanine, both into total liver trichloroacetic acid-precipitable protein and specifically into RBP, was determined during a 12-hra incubation period in each rat. Table II, column 1, shows that the animals in the three different retinol nutritional status incorporated almost identical amounts of [3H]leucine, [3H]lysine, and [3H]phenylalanine into total trichloroacetic acid-precipitable proteins, approximately 1.6 x 10^6 cpm/mg of liver protein. The portion of this total protein synthesis which corresponded to RBP was determined for each rat by immunoprecipitation of RBP with a specific rabbit anti-rat RBP antiserum.

Fig. 1 shows the migration pattern of immunoprecipitated [3H]-labeled proteins in a SDS-12.5% polyacrylamide gel. A peak of immunoprecipitated radioactivity was detected at approximately 20,500 daltons when rabbit anti-rat RBP antiserum was used. This coincident in migration with purified rat serum RBP. Immunoprecipitation with nonimmune serum resulted in only background radioactivity the entire

**TABLE I**

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Vitamin A</th>
<th>RBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Liver</td>
<td>Serum</td>
</tr>
<tr>
<td>μg/dl</td>
<td>μg/liver</td>
<td>μg/ml</td>
</tr>
<tr>
<td>Normal</td>
<td>56.7 ± 8.9</td>
<td>796 ± 236</td>
</tr>
<tr>
<td>Retinol-depleted</td>
<td>3.0 ± 1.2</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td>Retinol-repleted</td>
<td>73.0 ± 7.5</td>
<td>39.8 ± 17.9</td>
</tr>
</tbody>
</table>

Significantly different p < 0.01. Values with different superscripts are significantly different p < 0.01.

**TABLE II**

Effect of retinol status of rats on in vivo RBP and total protein synthesis in liver

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Radioactivity in total protein (1)</th>
<th>Radioactivity in RBP (2)</th>
<th>Relative rate of RBP synthesis (2/1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.64 ± 0.18</td>
<td>42.0 ± 2.6</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>Retinol-depleted</td>
<td>1.57 ± 0.14</td>
<td>41.4 ± 5.5</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>Retinol-repleted</td>
<td>1.60 ± 0.24</td>
<td>41.1 ± 0.4</td>
<td>0.26 ± 0.03</td>
</tr>
</tbody>
</table>

No significant differences found between treatments.
length of the gel. Finally, the addition of 50 µg of unlabeled purified serum RBP (10 times more than the estimated amount of RBP in the sample) to unlabeled purified serum RBP using the same amount of unlabeled serum RBP detected. This indicates that the immunoprecipitation reaction, as carried out, had both a high specificity for RBP and a limited capacity for specific immunoprecipitation.

The extent of incorporation of [³H]leucine, [³H]lysine, and [³H]phenylalanine into RBP in the three experimental groups is summarized in Table II, column 2. The relative rate of RBP synthesis (Table II, column 3) was calculated by dividing the amount of [³H]-labeled amino acids incorporated into RBP by that incorporated into total liver protein. No differences were found in the relative rate of RBP synthesis among the normal, retinol-depleted, and retinol-repleted rats (0.26 to 0.27% of total labeled protein detected as RBP).

Fig. 2 demonstrates the migration pattern of [³H]RBP immunoprecipitated from the rats in the three different retinol states. The immunoprecipitated [³H]RBP obtained from the normal, retinol-depleted, and retinol-repleted rats all migrated to the same position on the gel. The newly synthesized [³H]RBP in all three groups, therefore, had the same molecular weight, approximately 20,500.

Translatable Level of RBP-specific mRNA—Table III, columns 1 and 2, lists the amount of total RNA and poly(A⁺) RNA isolated per g of liver from the rats in the three different vitamin A (retinol) nutritional states. Although we tended to isolate less RNA and poly(A⁺) RNA per g of liver from the retinol-depleted rats, there were no statistically significant differences in this parameter found between the rats in the three different groups. As expected, the liver weight of the retinol-depleted rats was 20% lower than that of the normal rats.

Fig. 3 shows the translational activity of poly(A⁺) RNA prepared from the livers of normal, retinol-depleted, and retinol-repleted rats. The addition of poly(A⁺) RNA isolated from each of the three treatment groups resulted in the stimulation of [³S]methionine incorporation into trichloroacetic acid-precipitable protein which was approximately proportional to the amount of poly(A⁺) RNA added to the translation mixture over the concentration range of 0.25 to 1.5 µg of poly(A⁺) RNA/30 µl of lysate. Levels of poly(A⁺) RNA above 1.5 µg/30 µl of lysate resulted in no additional protein synthesis. There were no consistent differences observed in the ability of the poly(A⁺) RNA isolated from the normal, retinol-depleted, and retinol-repleted rats to direct the incorporation of [³S]methionine into trichloroacetic acid-precipitable protein.

Table III, column 3, lists the relative amount of translatable RBP-specific mRNA in the three groups of rats. Total poly(A⁺) RNA was translated in the rabbit reticulocyte cell-free protein-synthesizing system. The translatable level of
DISCUSSION

This study was designed to explore the effects of retinol depletion and repletion on the rate of RBP synthesis in liver. Since RBP specifically binds and transports retinol in plasma, it was of interest to study the role that the specific ligand might play in the control of RBP synthesis. Previous work has shown that retinol plays an important role in the regulation of RBP secretion from liver and in the maintenance of plasma RBP levels. Thus, in retinol deficiency, the secretion of RBP is specifically blocked; as a result, plasma RBP levels fall and liver RBP levels rise and plateau at an abnormally high value (6, 7). Repletion of vitamin A-depleted rats with retinol results in a rapid movement of RBP from the liver into plasma. This release of RBP was not blocked by inhibitors of protein synthesis, either puromycin or cycloheximide (7), suggesting that the secreted RBP came mainly from the expanded liver pool of RBP.

The block in RBP secretion seen after retinol depletion is highly specific for RBP. Thus, neither vitamin A depletion and deficiency nor retinol repletion of deficient rats significantly altered plasma levels of prealbumin (23). In both normal and vitamin A-deficient rats, RBP in the liver is found mainly associated with the liver microsomes (11) and particularly enriched in the rough microsomal fraction (1). Studies with isolated Golgi-rich fractions suggest that the Golgi apparatus and secretory vesicles are involved in the normal pathway of RBP secretion in the liver, and that the block in RBP secretion found in retinol deficiency occurs at a site before the RBP molecule reaches the Golgi (24).

The work reported here examined the question of whether retinol exerts an effect on RBP synthesis as well as on its secretion. Two kinds of studies were carried out. In the first study, the relative rate of hepatic RBP synthesis in vivo was measured and was found to be the same in normal, in retinol-depleted, and in retinol-repleted rats. In addition, the newly synthesized RBP in the retinol-depleted and -repleted rats appeared to have the same molecular weight as that in normal animals. In the second study, the relative level (activity) of translatable RBP-specific mRNA was measured by in vitro translation assays. Here, too, no differences were observed among the three groups. Taken together, these studies demonstrate that retinol status does not exert an effect on RBP synthesis rate, as it does on RBP secretion.

Since retinol-deficient rats show low levels of RBP in plasma and high levels in liver (see Table I), we had anticipated that these rats would show a reduced rate of RBP synthesis, secondary to the prolonged block in RBP secretion. The results presented here demonstrate that this was not the case, and that RBP synthesis rate remained normal in the retinol-depleted rats. In the retinol-deficient rat, RBP secretion is blocked, and liver RBP levels rise to a new, elevated steady state (6, 7). The rats employed in the present study demonstrated this phenomenon, as shown in Table I. The present finding that the RBP synthesis rate remained normal in the retinol-depleted rats suggests that RBP degradation rate in liver must have increased sufficient to maintain the new, elevated steady state levels. These considerations suggest that the process of RBP catabolism in the liver may play an important role in the overall metabolism of RBP in the liver and a significant regulatory role under certain conditions. No information is currently available about RBP degradation in liver.

In our studies of in vivo protein synthesis, a short 12-min pulse label period was used in order to prevent the loss of newly synthesized radioactively labeled RBP to the plasma. Studies reported by Schreiber et al. (12) demonstrated that 14C-labeled proteins could not be detected in the caudal blood of rats prior to 15 min after an injection of [14C]leucine. Similar results have been reported by Peters for albumin (13). In addition, this method has been successful in the study of several liver secretory proteins including α-globulin (25) and albumin (26). Therefore, it is likely that the in vivo experiment accounted for the complete amount of RBP synthesized since very little was lost from the liver through secretion.

The use of a translational assay for quantitating specific mRNA levels for any protein is subject to a certain degree of reservation. It is possible that different mRNA species may be translated with varying efficiencies in the "idealized" in vitro environment. Therefore, the relative amount of translatable RBP-specific mRNA may not accurately reflect the percent of mRNA that actually codes for RBP in the liver. However, comparison of the values for the relative translatable RBP-specific mRNA among the three different experimental groups should be quantitatively valid, especially since the poly(A) RNA samples from the three groups translated very similarly to each other in the reticulocyte lysate system.

In all three rat groups, the relative rate of RBP synthesis in vivo was approximately 0.26%, whereas the relative translatable level of RBP-specific mRNA was about 0.14%. At least three factors might have contributed to this difference. First, the efficiency of translation of given mRNA species might be different in vivo as compared to the "optimized" in vitro system. Second, different labeled amino acids were used in the two determinations (leucine, lysine, and phenylalanine for the in vivo study and methionine for the in vitro translation assays). Different relative contents of these different amino acids in RBP as compared to other synthesized proteins would contribute to the observed quantitative difference. Finally, the fact that the labeled product synthesized in vivo appeared to be mature RBP, whereas the product of the translation assays was pre-RBP might also have been a contributing factor.

The lack of a response of RBP synthesis rate and translatable level of RBP mRNA to changes in retinol status is unusual when compared to other proteins for which information is available. RBP appears to differ from other binding proteins, whose synthesis is controlled by their specific ligands. Examples include transferrin (27, 28), ferritin (29), and zinc metallothionein (30). RBP also differs from enzymes such as phosphoenolpyruvate carboxykinase (31), malic enzyme (32), fatty acid synthetase (33), 6-phosphogluconate dehydrogenase (34), and glucose-6-phosphate dehydrogenase (35) whose activity, synthesis, and mRNA levels have been shown to be influenced by dietary changes which alter their specific substrate levels. Thus, the control of RBP synthesis is unusual in not being influenced by retinol, the molecule with which RBP specifically and functionally interacts. Further information is needed to define the mechanism involved in the retinol-mediated post-translational regulation of RBP secretion.

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