Ultrastructural localization of plasma retinol-binding protein in rat liver

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Abstract Immunocytochemical studies were carried out to examine the subcellular localization of plasma retinol-binding protein (RBP) in rat liver. The studies used normal, retinol-deficient, and retinol-repleted retinol-deficient rats with or without colchicine pretreatment. Affinity-purified monomeric Fab' fragments from the IgG fraction of rabbit anti-rat RBP were conjugated to horseradish peroxidase. This conjugate effectively penetrated into tissue sections and enabled RBP to be localized by high resolution immunoelectron microscopy. In the normal liver parenchymal cell, RBP was found to be localized in the synthetic and secretory structures including endoplasmic reticulum (ER), Golgi complex (GC), and secretory vesicles. With the method used, significant localization of RBP was not observed in hepatic cells other than parenchymal cells. The distribution of RBP-positive areas within parenchymal cells changed markedly with retinol depletion. Thus, a heavy accumulation of RBP in the ER, accompanied by a marked decrease of the RBP-positive GC and secretory vesicles, was demonstrated in liver parenchymal cells from retinol-deficient rats. After repletion of deficient rats with retinol, the RBP that accumulated in the ER appeared to move rapidly from the ER through GC and secretory vesicles to the cell surface. Pretreatment with colchicine led to marked increase in RBP-positive secretory vesicles in retinol-repleted rat liver parenchymal cells. The results reported here demonstrate that the specific block in hepatic RBP secretion seen in retinol deficiency involves an inhibition of the movement of RBP from the ER to the GC in the parenchymal cell. — **Suhara, A., M. Kato, and M. Kanai.** Ultrastructural localization of plasma retinol-binding protein in rat liver. *J. Lipid Res.* 1990. **31:** 1669–1681.

Supplementary key words immunoelectron microscopy • protein secretion • colchicine

Vitamin A is transported normally in postabsorptive plasma as the lipid alcohol retinol bound to a specific transport protein, plasma retinol-binding protein (RBP). The isolation and partial characterization of RBP was first reported in 1968 (1). Since then, extensive studies have provided a considerable amount of information about the structure, metabolism, and biological roles of this protein (see references 2 and 3 for recent reviews).

RBP is a single polypeptide chain with a molecular weight close to 21,000, which has a single binding site that selectively binds one molecule of retinol (holo-RBP). It is synthesized mainly in the liver and secreted into plasma where it interacts strongly with another protein, plasma transthyretin (TTR, mol wt approximately 55,000), and normally circulates as a 1:1 molar RBP-TTR complex (1, 2). The association of retinol with RBP and the binding of RBP to TTR increases the stability of retinol and prevents ready filtration of RBP through renal glomeruli. After delivering retinol to its target organs, the resulting apo-RBP has a reduced affinity for TTR. RBP not complexed with TTR is readily filtered by the renal glomeruli, reabsorbed in the renal proximal tubules, and is catabolized (4, 5).

Vitamin A mobilization from the liver is highly regulated by factors that control the rates of hepatic RBP synthesis and secretion. Retinol deficiency specifically inhibits the secretion of RBP by the liver, leading to a decrease in the plasma level of RBP and the concomitant increase of RBP (as apo-RBP) in the liver (6–8). Upon retinol repletion, RBP is rapidly secreted from the expanded liver pool into the plasma as holo-RBP (7, 8). Some information is available about the cellular and molecular mechanisms that mediate these phenomena. The specific subcellular site and the molecular mechanism whereby retinol regulates RBP secretion remains, however, to be elucidated.

The drug colchicine has been shown to inhibit the secretion of several plasma proteins that are synthesized in the liver (9–11). The inhibition appears to occur at a site between the formation of Golgi-derived secretory vesicles and their fusion to the plasma membrane (10, 12, 13), and may be related to the disruption of microtubules induced by colchicine (14). A previous report has demonstrated

Abbreviations: BiP, binding protein; DAB, 3,3'-diaminobenzidine; ER, endoplasmic reticulum; GC, Golgi complex; HRP, horseradish peroxidase; NE, nuclear envelope; RBP, retinol-binding protein; SC, perisinusoidal stellate cell; TTR, transthyretin; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography.

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that colchicine inhibits the retinol-stimulated secretion of RBP by the liver (15).

Adding to these biochemical studies, immunocytochemical studies have demonstrated the cellular localization of RBP in rat liver, kidney, and pancreas (16, 17), and in human kidney and pancreas (18, 19). As yet, however, the subcellular localization of RBP has been assessed mainly by cell fractionation techniques (20–23). We now report studies on the direct demonstration of the subcellular localization of RBP in liver using highly specific peroxidase-conjugated anti-rat RBP Fab'. These studies provide new information about the processes of RBP synthesis, secretion, and its regulation by retinol in the liver.

METHODS

Animals and experimental protocols

Male weanling Sprague-Dawley rats purchased from Charles River Japan (Shizuoka, Japan) were fed a vitamin A-deficient diet (24) for 5 weeks and then the same diet supplemented with 12 mg of retinoic acid per kg of diet for 2 weeks to maintain good health. During the week before the experiment, the rats were again fed the unsupplemented vitamin A-deficient diet. The control rats were fed the identical diet supplemented with 4 mg retinol (in peanut oil) per kg diet. On the 9th week all rats were killed for the experiments.

Vitamin A-rich chylomicrons were prepared as described elsewhere (8). Briefly, thoracic duct cannulation was performed on a rat fed a normal chow diet. Several times the rat was given 0.5 ml of peanut oil containing 1.5 mg retinol. Collected chyle was layered under saline and centrifuged at 25,000 rpm for 25 min. The packed chylomicrons at the top of the centrifuge tube were collected and redispersed in saline. The total retinoyl (retinyl ester + retinol) concentration of the chylomicron preparation was determined.

The study was carried out in four groups, each consisting of two rats: *i*) control rats; *ii*) retinol-deficient rats; *iii*) retinol-deficient rats repleted with retinol; *iv*) retinol-deficient rats repleted with retinol after prior treatment with colchicine. Before the experiments, rats were fasted overnight with with free access to water. In the study of retinol repletion, 1 ml of the chylomicron suspension containing 150 μg of retinol was injected via tail vein. Rats were killed after each of three time intervals: 15, 30, and 90 min. One rat was used to monitor the serum and tissue levels of vitamin A and RBP, and the other for the histochemistry; from the latter, blood samples were also obtained.

To explore the effects of colchicine on the secretion of RBP in the liver, retinol-deficient rats were each given an intraperitoneal injection of colchicine (5 mg per kg body weight) 3 h prior to the chylomicron injection (13). Ninety minutes after retinol repletion the rats were killed for the histochemical study.

Rat RBP and preparation of the HRP conjugated Fab'

Preparation of the RBP and its antibody was described previously (16). The rabbit IgG fraction was digested with pepsin (Sigma, St. Louis, MO), and $F(ab')_2$ fragments specific for RBP were obtained by immunosorbent affinity chromatography with RBP coupled to Sepharose 4B (Pharmacia). Fab' fragments specific for RBP were then obtained by the reduction of $F(ab')_2$ with 2-mercaptoethylamine (Sigma). The Fab' was conjugated to horseradish peroxidase (HRP) (Toyobo, Tokyo, Japan) monomerically by using succinimidyl 6-maleimidehexanoate (Dojindo Lab., Kumamoto, Japan). This conjugation technique has been described elsewhere in detail (25). For control studies, nonspecific Fab' fragments were obtained from nonimmunized rabbit serum and conjugated to HRP by the same method.

Purity of the RBP and the specificity of the HRP-Fab'

Purified RBP was analyzed by SDS-PAGE using the buffer system of Laemmli (26) with a 12% acrylamide gel. The specificity of the anti-RBP Fab'-HRP was determined by modifications of the electrophoretic blotting technique described by Towbin, Staehelin, and Gordon (27). The blotted membrane was first treated with 1% BSA in PBS containing 0.05% Tween 20 (PBS-Tween) to block nonspecific protein binding, then incubated with HRP-Fab' anti-RBP (0.4 nM) for 2 h at room temperature. The membrane was washed with PBS-Tween several times and reacted with 0.1% 3,3'-diaminobenzidine (DAB) in 50 mM Tris-HCl buffer, pH 7.6, containing 0.003% H_2O_2 for 5 min.

Immunoelectron microscopy

The rats were anesthetized with diethyl ether. The livers were fixed by perfusion with 4% paraformaldehyde fixative according to Yokota and Fahimi (28). Thick sections were cut at approximately 50 μm by Vibratome (Polaron, U.K.), and then treated with 0.1 M lysine in 0.15 M cacodylate buffer, pH 7.4, for 30 min. After brief washing with PBS containing 0.15 M sucrose (PBS-Suc), tissue sections were incubated in HRP-Fab' solution (50 μg/ml HRP-Fab' anti-rat RBP, 0.005% Triton X-100, in PBS) overnight at 4°C with continuous shaking. After incubation they were washed extensively with PBS-Suc and refixed with 2% glutaraldehyde in PBS for 30 min at 4°C. The sections were washed again with PBS-Suc several times, and then incubated in DAB/H_2O_2 medium (29) for 5 min at room temperature. The sections were then postfixed with 1% OsO_4 in PBS for 60 min, and processed for embedding in Epon 812. The ultrathin sections were cut with a diamond knife and mounted on grids. Grids were stained with lead citrate alone and observed by electron microscope (Hitachi HS-9, Japan). Control sections were incubated with nonspecific Fab'-HRP, processed by the

same procedures, and stained with both uranyl acetate and lead citrate.

Determination of RBP and vitamin A

Serum and tissue RBP levels were measured by enzyme-linked immunosorbent assay (ELISA). To measure tissue RBP, each tissue was homogenized in 5 volumes of PBS containing 0.1% Triton X-100. After centrifugation, the supernatant was stored at −20°C until assayed. Samples were diluted with PBS-Tween, 1:10,000 for serum and 1:2,000 for tissue sample, respectively. The assays were performed using 96-well microplates as follows: *a*) 200 μl of 50 mM sodium bicarbonate buffer, pH 9.5, containing 10 μg of affinity-purified IgG from rabbit anti-rat RBP, overnight at 4°C; *b*) 200 μl of standard substances or diluted samples for 2 h at room temperature; *c*) 200 μl of 0.86 ng/ml HRP-Fab' anti-rat RBP in PBS-Tween for 2 h at room temperature; and *d*) 200 μl of 0.04% *O*-phenylenediamine, 0.003% H_2O_2, in 0.05 M phosphate citrate buffer, pH 5.0, for 1 h at room temperature. Between each step wells were washed with PBS-Tween. After the last step the reaction was stopped by adding 50 μl of 1 N H_2SO_4, and absorbance at 492 nm was measured. All procedures were performed by the ELISA processor II (Behring, West Germany). Sample concentration was calculated by the standard curve obtained on each plate. The precision of the assay was monitored by the measurement of pooled normal rat serum in each assay.

Serum and tissue retinoid (retinyl ester and retinol) levels were measured by isocratic reversed phase HPLC. All procedures were performed under dim light. The samples were lyophilized to dryness and the residues were extracted as described previously (30). All HPLC work was performed on a Shimadzu LC-6A system (Shimadzu Co. Ltd., Kyoto, Japan), with detection wave length at 325 nm. The column used was 6.0 mm diam × 15 cm long, Shimpack CLC-ODS (Shimadzu), and the elution solvent was methanol–acetonitrile–chloroform 25:60:15 (31). All chromatography was done at ambient temperature with a flow rate of 1.0 ml/min. Standard substances (all-*trans*-retinol, retinyl acetate, retinyl palmitate) were purchased from Sigma. All chemicals were HPLC grade.

RESULTS

Antibody characterization and specificity

To determine the purity of the isolated RBP and the specificity of the HRP-Fab' conjugate of rabbit anti-rat RBP, SDS-PAGE and Western blotting were performed. As seen in **Fig. 1**, a single band of protein was obtained with purified RBP (lane 2) on SDS-PAGE. The Fab' conjugate detected the RBP on the transferred membrane from purified RBP (lane 4) and from normal rat serum

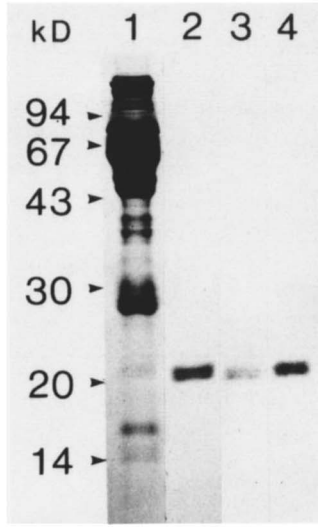

Fig. 1. Purity of the rat RBP and the specificity of the HRP-Fab' anti-rat RBP was assessed by SDS-PAGE and Western blot. Lanes 1 and 2: SDS-PAGE analysis of normal rat serum (lane 1) and purified rat RBP (lane 2). The gel was stained with Coomassie Blue. Lanes 3 and 4: Western blot analysis by the HRP-Fab' anti-rat RBP. Proteins were separated on an SDS-PAGE gel, electrophoretically blotted onto nitrocellulose, and the membrane was incubated with HRP-Fab' anti-rat RBP and reacted with DAB. Lane 3; normal rat serum; lane 4; rat RBP.

(lane 3). Nonspecific staining with the HRP-Fab' conjugate was not observed. These data verify the purity of RBP and the specificity of the HRP-Fab' anti-rat RBP.

Assay of RBP and vitamin A

Fig. 2 shows a standard curve of the ELISA for RBP, and the high sensitivity (0.4 ng/ml) obtained in this assay. The precision of the assay was high (inter-assay coefficient of variance = 4.6%, n = 5; intra-assay CV = 5.6%, n = 6). Both serum and tissue RBP levels were determined in this assay.

Table 1 shows the levels of serum and tissue RBP and total retinol (vitamin A) in the rats used for the present experiments. As expected, the retinol-deficient rats had serum RBP levels that were decreased to one-fourth of the normal value, with negligible serum and liver retinol levels. Also as expected, liver RBP levels were elevated about sixfold in the retinol-deficient rats. Rapid secretion of RBP from the liver occurred after retinol repletion, which was shown by the concomitant increase of plasma RBP and decrease of liver RBP. The quantitative analyses of RBP and retinol of these experimental animals thus confirmed the previous biochemical data in similar experimental protocols (6, 8, 15).

Subcellular localization of RBP in the normal rat liver

With the methods used in this study, the ultrastructure of the cells and antigenic reactivity of RBP were well pre-

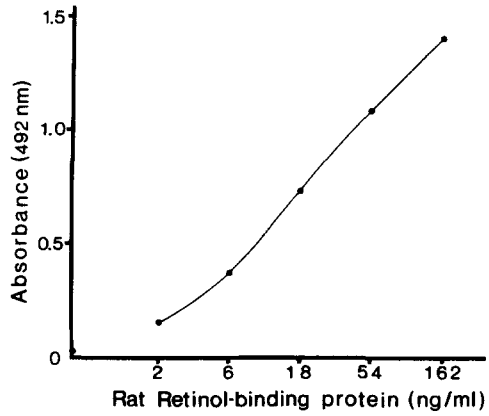

Fig. 2. Standard curve of the ELISA for rat RBP. Sandwich type ELISA was used to determine serum and tissue RBP levels. Assay procedure is detailed in Materials and Methods. The use of HRP-Fab' anti-rat RBP provided high sensitivity (0.4 ng/well).

served, and enabled us to visualize precisely the intracellular localization of RBP. Immunoreactive RBP localization was detected as an electron-dense deposit, consisting of osmiophilic DAB reaction products produced with HRP. Lipoprotein particles were observed as moderate electron-dense homogenous particles.

Specific staining of RBP was observed in all parenchymal cells in the liver in normal rats (**Fig. 3**). The most conspicuous deposits of reaction product were seen in the Golgi complex (GC). There was moderate specific immune RBP staining (Fig. 3) in the nuclear envelope (NE), rough endoplasmic reticulum (rER), smooth endoplasmic reticulum (small arrows), and in a few secretory vesicles (arrow heads). Nucleoplasm (N), mitochondria (M), and peroxisomes (P) were consistently negative. Control sections that were incubated with nonimmunized rabbit Fab' conjugated with HRP did not show any specific staining (see Fig. 5A); in these control sections the GC (asterisk), ER, and NE lumen did not shown any electron-dense deposits. Moderate electron-dense lipoprotein particles were observed in the Golgi stack. Some contents of lysosomes and intramitochondrial granules within the mitochondrial matrix were observed as electron-dense in both control sections (Ly and arrow heads in Fig. 5A) and sections specifically stained for RBP (Fig. 3). Glycogen granules also showed high electron density in the control section (G in Fig. 5A).

In the Golgi regions of sections specifically stained for RBP, the reaction product was present in the stacked Golgi cisternae and their peripheral dilatations (Figs. 3A, 3C). There were different staining patterns of GC. GC in

TABLE 1. Retinol and RBP levels in serum and liver

	Serum		Liver[a]	
Rat No.	Retinol[b]	RBP	Retinol[b]	RBP
	μg/dl	*μg/ml*	*μg/g*	
Control				
1	34.6	48.5	99.5	29.9
2	27.9	53.5	NA	NA
Retinol-deficient				
1	2.4	15.5	0.5	197.4
2	2.7	12.8	NA	NA
15 min after retinol repletion				
1	65.4	90.5	1.1	115.1
2	72.8	83.2	NA	NA
30 min after retinol repletion				
1	70.8	111.8	2.1	109.6
2	81.5	104.3	NA	NA
90 min after retinol repletion				
1	49.4	97.6	3.4	45.3
2	53.8	102.5	NA	NA

Serum and liver retinol and RBP were measured by HPLC and ELISA, respectively, as described in Materials and Methods; NA, not available.
[a]Per gram wet tissue.
[b]Retinol + retinyl ester

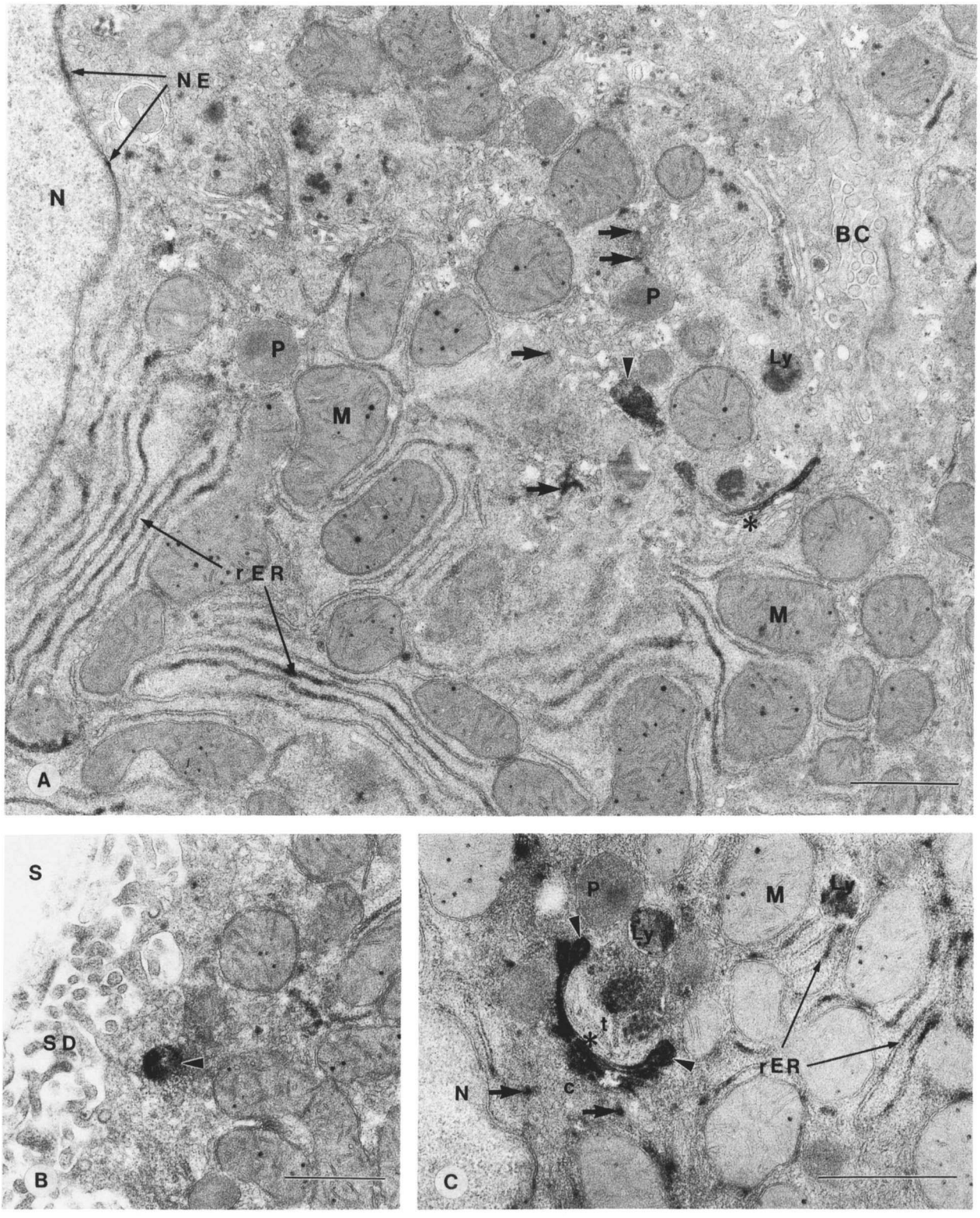

Fig. 3. Immunocytochemical localization of RBP in a normal liver parenchymal cell. Prominent electron-dense reaction products are observed in the Golgi complex (asterisk in 3A, and 3C), and in the endoplasmic reticulum both rough (rER) and smooth (arrows in 3A and 3C), including nuclear envelope (NE). Nucleoplasma (N), mitochondria (M), and peroxisomes (P) are negative. The reaction product for RBP is seen focally in cisternae of the rER, which is interposed with negative segments (3A, 3C). Golgi complex is positive for RBP in its stacked cisternae (3A, 3C). Golgi-derived secretory vesicles (arrow heads in 3A and 3C) are seen to contain RBP-positive reaction products, which seem to bud off from the terminal dilatations. Secretory vesicle is also observed occasionally under the sinusoidal border (3B). These secretory vesicles may contain heterogeneous material other than RBP which are observed as negative relief. Lysosomes (Ly) are sometimes observed containing electron-dense material. BC, bile canaliculi; M, mitochondria; S, sinusoid; SD, space of Disse; c, *cis*; t, *trans* side of Golgi complex. A: x 18,000; B: x 17,100; C: x 23,400; bars = 1 μm.

Fig. 3A showed labeling in the *trans* part of the stacked cisternae, whereas GC in Fig. 3C was labeled in the *cis* part. The *trans* side of the Golgi stacks tended to be stained most frequently.

Golgi-derived secretory vesicles were seen at the *trans* side of the GC (Figs. 3A, 3C; arrow heads). They often contained lipoprotein particles that were recognized as negative relief (Figs. 3A–3C; arrow heads). These secretory vesicles seemed to bud off from terminal dilatation of Golgi stacks (Figs. 3A, 3C; arrow heads). Beneath the sinusoidal lining, secretory vesicles containing RBP (arrow head in Fig. 3B) were occasionally found.

Focal specific staining for RBP was found in the cisternae of the rough and smooth ER including the nuclear envelope (Figs. 3A, 3C). There were consistently negative segments of ER interposed between the positive regions (Figs. 3A, 3C).

Concerning perisinusoidal stellate cells (SCs), there was no specific staining for RBP in these cells (**Fig. 4**). We could not observe any reaction products for RBP in ER or NE. There were electron-dense deposits around some lipid droplets of the cell (arrow heads in Fig. 4), which associated with surrounding membrane. However, this deposit was also observed in the control specimen nonspecifically stained with nonimmune Fab'-HRP (**Fig. 5B**). There was another type of lipid droplets which did not have this deposit (asterisks in Fig. 4). We could not find any organelles showing a distinct reaction product for RBP in stellate cells, nor in other nonparenchymal cells such as Kupffer cells and endothelial cells.

Subcellular localization of RBP in the retinol deficient rat liver

Fig. 6 shows the localization of RBP in the liver parenchymal cells of a retinol-deficient rat. The total extent of specific immune staining for RBP was markedly increased in the retinol-deficient liver, consistent with the increased content of RBP that is seen in such livers. The reaction

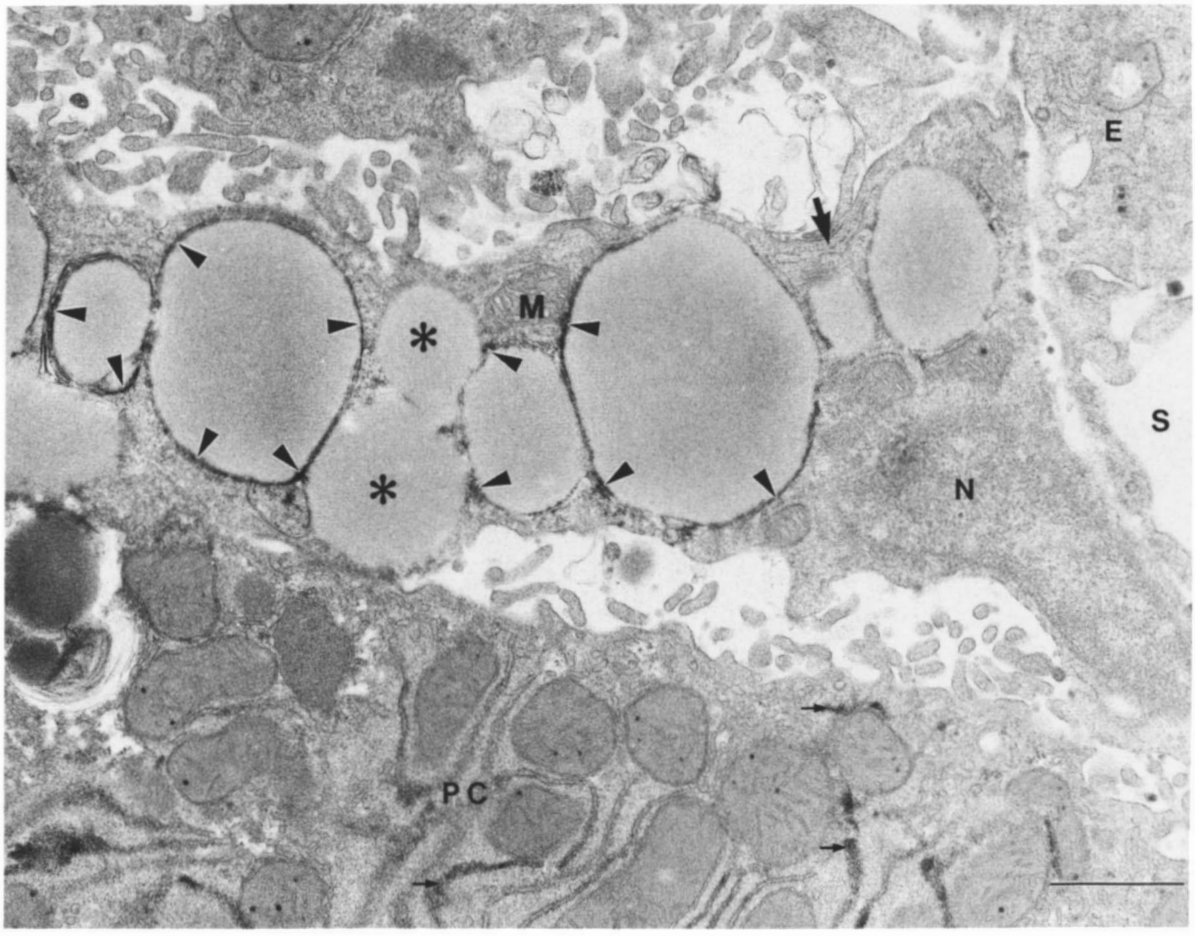

Fig. 4. Immunocytochemical localization of RBP in perisinusoidal stellate cell in normal rat liver. There are numerous lipid droplets that are surrounded by electron-dense deposits (arrow heads). These droplets are Type I lipid droplets, according to the classification of Wake (53). Droplets with asterisks do not have these deposits and do not have surrounding membrane; they are type II lipid droplets (53). No specific staining for RBP was observed in any organelles such as ER (arrow), and nuclear envelope. The ER (small arrows) in adjacent parenchymal cell (PC) is positive for RBP. E, endothelial cell; M, mitochondria; N, nucleus; S, sinusoid; × 17,500; bar = 1 μm.

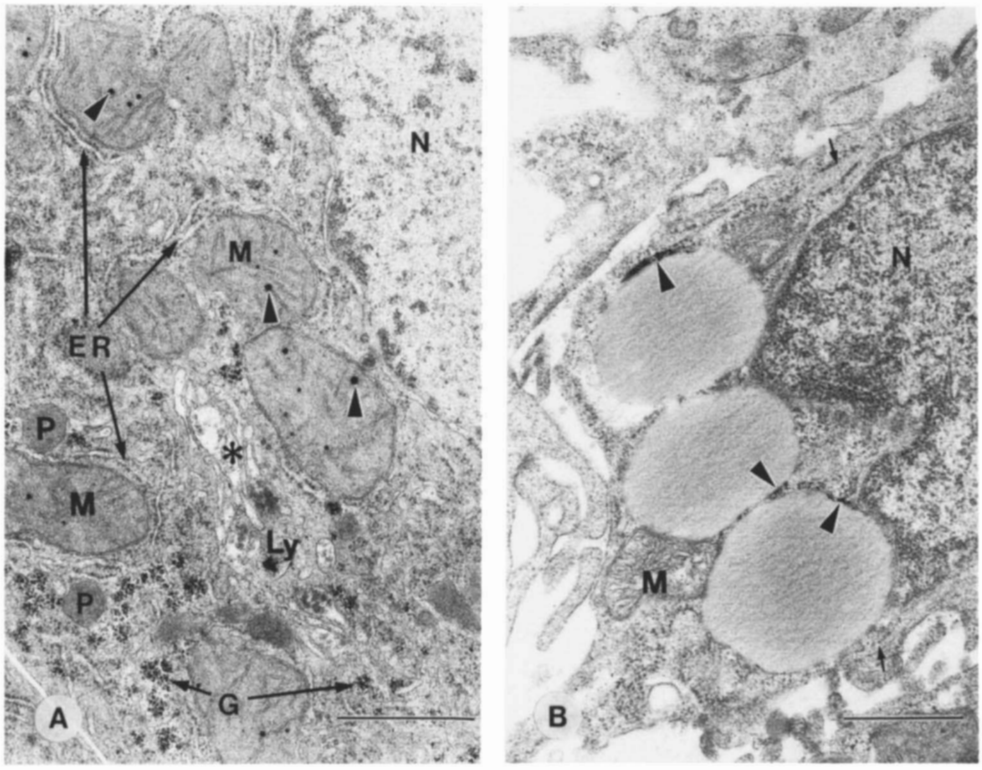

Fig. 5. Control preparations incubated with nonimmune (nonspecific) Fab' labeled with HRP. The rat liver parenchymal cell (Fig. 5A) shows no specific staining in the Golgi complex (asterisk), ER, or other cell organelles. Moderate electron-dense lipoprotein particles are observed in the Golgi stack. Electron-dense mitochondrial granules (arrow heads) are observed. Glycogen granules (G) are also recognized as electron-dense particles. Lysosome (Ly) has an electron-dense inclusion. The perisinusoidal stellate cell (Fig. 5B) contains lipid droplets that possess an electron-dense deposit (arrow heads). Small arrows indicate ER. No other organelles are positive for electron-dense staining. M, mitochondria; N, nucleus; P, peroxisome. A: × 18,000; B × 15,870; bars = 1 μm.

products were prominently increased in the lumen of both rough and smooth ER and in the nuclear envelope (rER, sER, and arrow heads in Fig. 6). On the other hand, the Golgi vacuoles and stacked Golgi cisternae were devoid of specific staining for RBP (Fig. 6 inset and asterisks in Fig. 6).

Effect of retinol repletion on the retinol-deficient rat liver

Fig. 7 shows the localization of RBP in parenchymal cells that was observed 15 min (Fig. 7A), 30 min (Fig. 7B), and 90 min (Figs. 7C, D) after the intravenous repletion of retinol-deficient rats with retinol-rich chylomicrons. The most striking feature of the events caused by retinol repletion was a rapid migration of the accumulated RBP from the ER into secretory organelles, including numerous vesicular structure (small arrows in Figs. 7A, B), the GC (asterisks in Figs. 7A, B, and 7D), and secretory vesicles (arrow heads in Figs. 7C, D). Immunoreactive RBP was already seen in the secretory apparatus as early as 15 min after retinol repletion (Fig. 7A). Almost all stacks of GC showed positive reaction for RBP by 90 min after retinol repletion (asterisks in Fig. 7D).

Effect of colchicine on the retinol-repleted retinol-deficient rat liver

In the parenchymal cells of the retinol-deficient rat liver treated with colchicine 3 h before retinol repletion (**Fig. 8**), numerous secretory vesicles that showed reaction products were observed (V in Fig. 8), although Golgi-stacks were less labeled for RBP compared to retinol-repleted state (Fig. 8 inset). Some secretory vesicles were localized near the sinusoidal border. There were still some RBP found in the ER.

DISCUSSION

The present studies provide detailed ultrastructural information on the subcellular immunocytochemical localization of RBP within the rat liver parenchymal cell, and about the anatomic site within the cell where retinol deficiency inhibits RBP secretion. The use of a maleimide compound for the conjugation of HRP to the hinge por-

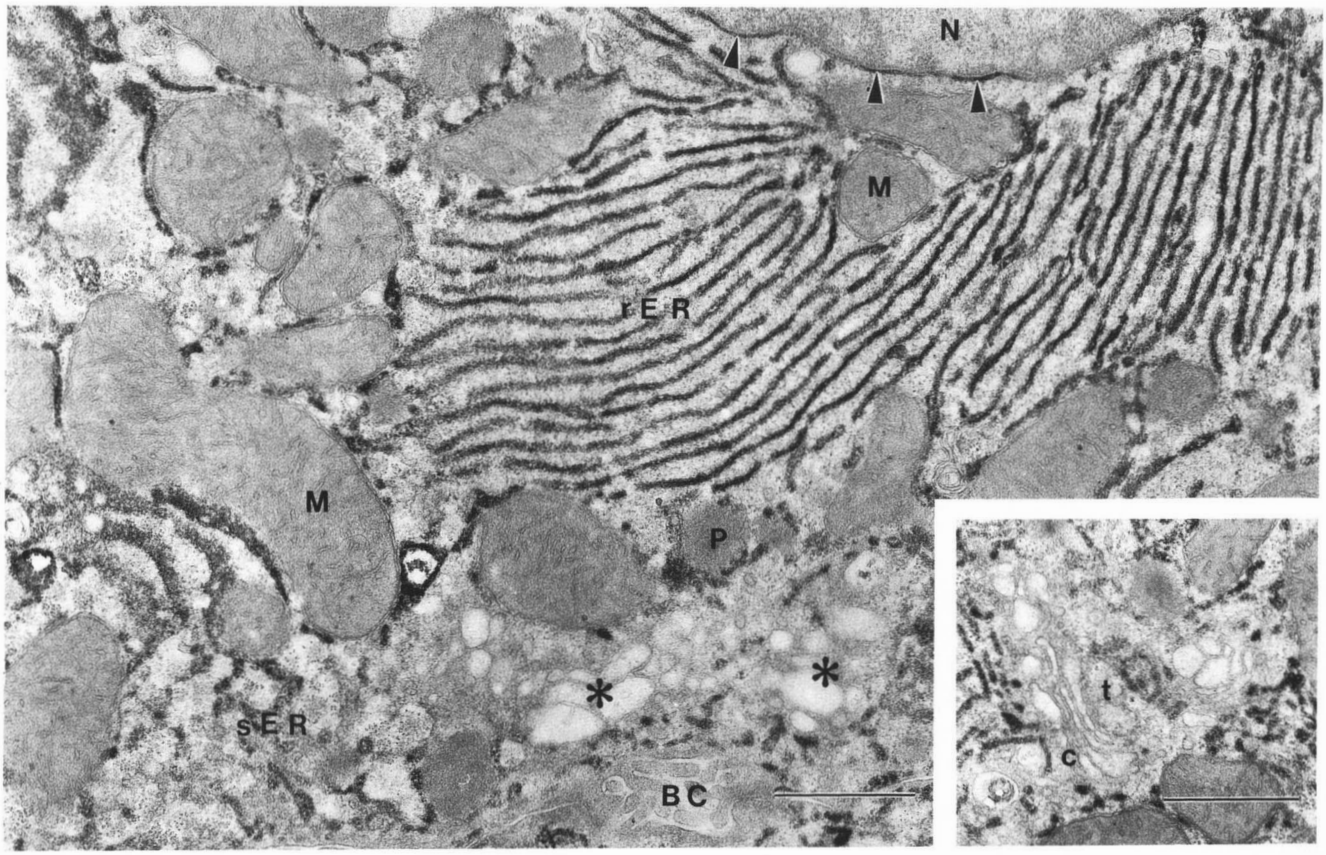

Fig. 6. Immunocytochemical localization of RBP in the retinol-deficient rat liver parenchymal cell. A prominent difference of RBP localization is observed compared to normal liver. ER including nuclear envelope (arrow heads) is much more intensely filled with reaction product for RBP, and most Golgi complexes (asterisks, Golgi saccules; inset, Golgi stacks) are free of reaction products. Mitochondria (M) and peroxisomes (P) are negative. BC, bile canaliculi, N, nucleus; rER, rough endoplasmic reticulum; sER, smooth endoplasmic reticulum; c, *cis*; t, *trans* side of Golgi complex. × 18,900; inset, × 18,000; bars = 1 μm.

tion of Fab' fragment of IgG enabled us to explore RBP localization with high resolution immunoelectron microscopy. The key features of this method are observations that the monomeric conjugate has better penetration into the relatively well preserved tissue sections, and that the variable region of the Fab' fragment, which recognizes the antigen (here, RBP), is fully unoccupied. The use of an antibody purified by immunosorbent affinity chromatography effectively avoids background and nonspecific staining, as discussed previously (19). The only drawback of this method is that since the reaction product is detected as an electron-dense deposit, we cannot localize the protein in organelles that are already electron-dense in the control specimen. Thus, for electron-dense organelles, such as glycogen granules, mitochondrial granules, osmiophilic lipid droplets, and lysosomes, it is not possible to say with certainty whether the electron-dense deposits are due to RBP presence or to the staining properties of these organelles. This is especially true for hepatic lysosomes which often contain electron-dense materials, and thus it is difficult to rule out positive immuno-reaction for the protein (in this case, RBP) which is degraded intracellularly in the lysosome. However, this conjugation technique should prove broadly useful for a variety of immunoelectron microscopic studies, when the post-embedding immunoelectron method significantly diminishes antigenicity of the protein.

The liver plays a critical role in the metabolism of vitamin A in the body. Vitamin A is mobilized from the liver as retinol bound to RBP. Secretion of RBP from the liver is highly regulated by the nutritional vitamin A status of the animal. The synthesis and secretion of RBP and the regulation of RBP secretion by retinol have been studied both in vivo (6, 7, 32), and in vitro (33–35).

The results reported here confirm the previous findings at the level of the light microscope, using immunofluorescence microscopy (36) and the unlabeled immunohistochemical method (16), that RBP is localized specifically in rat liver parenchymal cells. In the latter study (16), a marked increase in intensity of the specific diffuse staining for RBP was observed in parenchymal cells of the retinol-deficient liver.

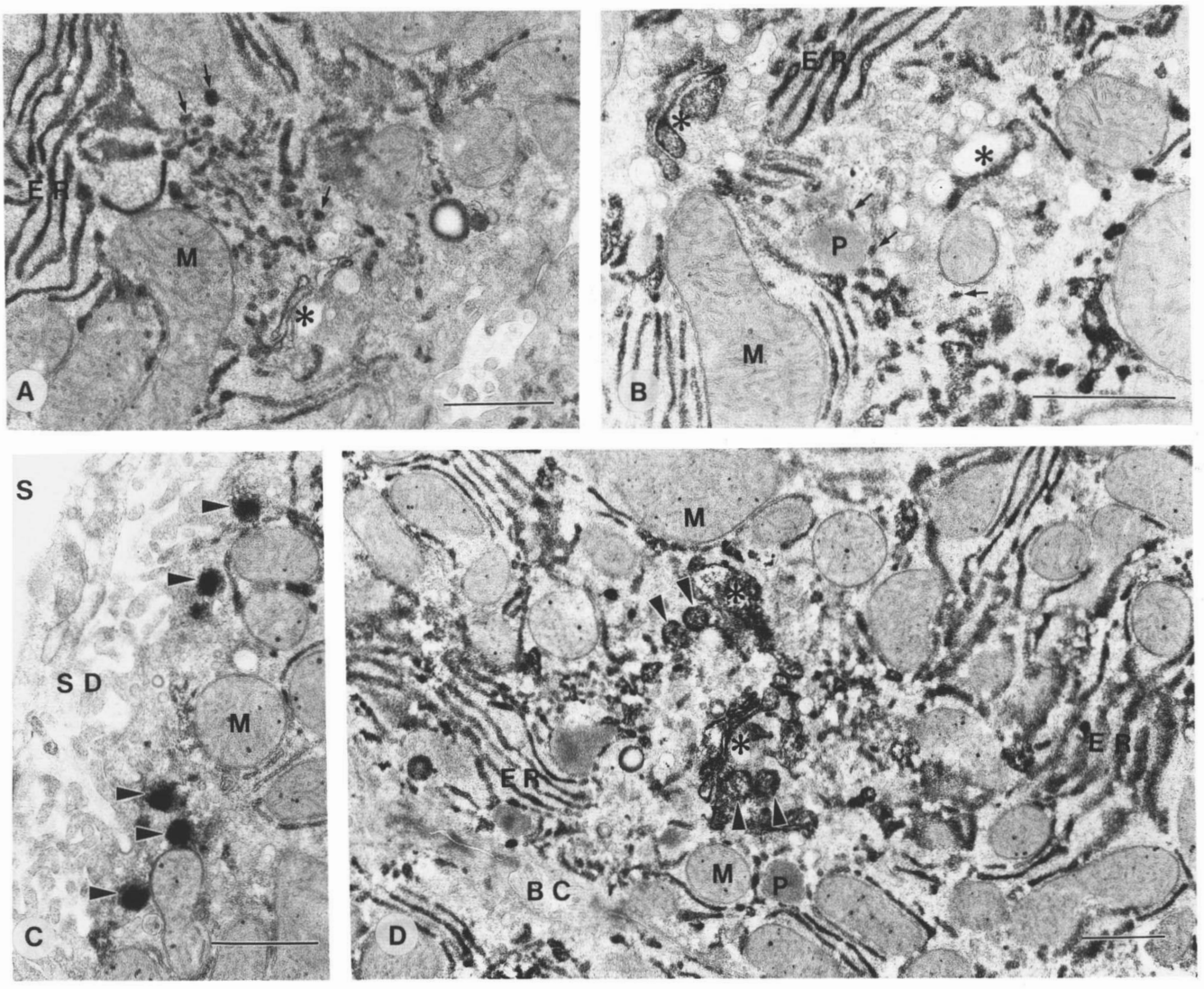

Fig. 7. Immunocytochemical localization of RBP in the retinol-deficient rat liver parenchymal cell at various times after retinol repletion. After 15 min (Fig. 7A), the Golgi complex (asterisk) is positive for RBP, and numerous vesicular structures positive for RBP are noted (small arrows). After 30 min (Fig. 7B), Golgi complexes (asterisks) are more clearly stained for RBP, and positively stained vesicular structures are also noted (small arrows). Ninety min after repletion (Fig. 7C and 7D), there are numerous secretory vesicles (arrow heads) strongly stained for RBP beneath the sinusoidal lining (Fig. 7C) and in the Golgi region (asterisks in Fig. 7D). ER, endoplasmic reticulum; M, mitochondria; N, nucleus; P, peroxisome; S, sinusoid; SD, space of Disse. A: × 15,750; B: × 21,000; C: × 15,000; D: × 12,000; bars = 1 μm.

In the present study, we observed the localization of immunoreactive RBP in all of the subcellular structures that comprise the synthetic and secretory apparatus of the liver parenchymal cells. In the normal state, RBP was localized in the NE, ER, GC, and secretory vesicles. The subcellular localization of RBP was generally similar to that which has been reported for albumin (28). Neither RBP nor albumin contain a sugar residue; nevertheless, after their synthesis in the ER, both of these proteins are sorted through the GC and then transported via secretory vesicles to the cell surface.

In the retinol-deficient rat liver, although RBP secretion is markedly inhibited, RBP mRNA levels and RBP synthesis rate are unchanged from those seen in normal animals (37, 38). It is clear that retinol deficiency specifically inhibits the secretion, and not the synthesis of RBP. As a consequence, in the deficient rat, the synthesized RBP must accumulate in some organelles of the parenchymal cells. Previous biochemical findings by cell fractionation showed that RBP is accumulated in the ER in retinol deficiency, and the site of blockage of RBP secretion was postulated to be mainly between the ER and GC (22, 23, 24), or between the *cis* and *trans* stacks of GC (21). The results reported here show that in the retinol-deficient state, a considerable amount of RBP accumulates in the lumen of the ER and the GC becomes depleted of RBP. Moreover, numerous vesicular structures and the GC rapidly develop specific immune staining for RBP after

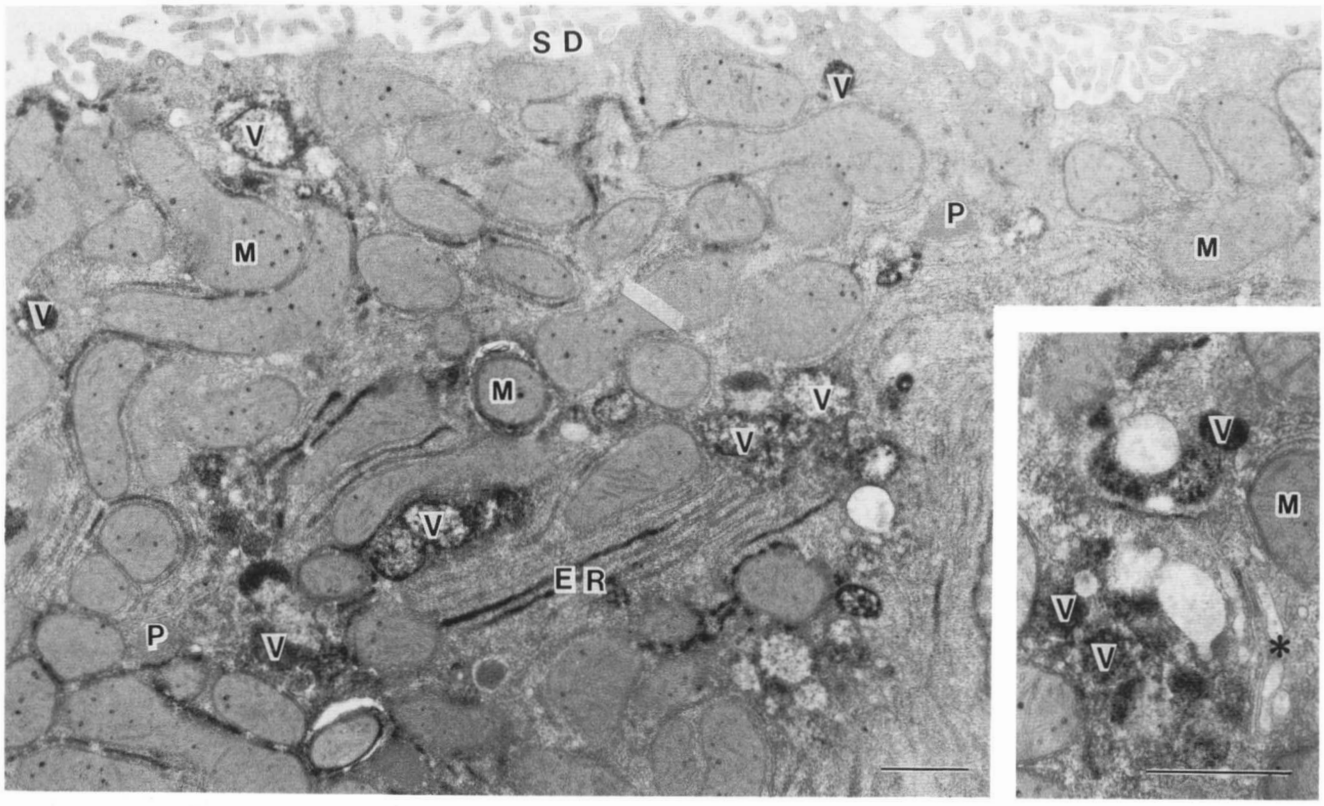

Fig. 8. Immunocytochemical localization of RBP in the liver parenchymal cell of the retinol-deficient rat treated with colchicine 3 h prior to retinol repletion. The rat was killed 90 min after retinol repletion. Numerous secretory vesicles (V) that contain reaction product for RBP are noted, in the Golgi region (inset), beneath the sinusoidal border, and in other areas. ER, endoplasmic reticulum; M, mitochondria; P, peroxisome; SD, space of Disse. × 12,000; inset, × 19,000; bars = 1 μm.

retinol repletion. These data provide definitive evidence that retinol regulates RBP secretion by regulating the transfer of RBP from the ER to the GC. Additional biochemical data that other plasma proteins, such as albumin and ceruloplasmin, do not increase in the ER and are secreted normally in the vitamin A-deficient state (23) and that a rate-limiting step of protein secretion exists between the ER and the GC (20, 39) suggest that RBP intracellular transport at this site from the ER to the GC is highly regulated by its ligand retinol. Thus it may be more precise to conclude that in retinol deficiency, the selective blockage of RBP secretion from the liver parenchymal cells is caused by a lack of trigger (retinol) needed for the release of RBP from the ER into transfer vesicles.

There is a considerable amount of information available about protein sorting in the ER in relation to immunoglobulin heavy-chain binding protein (BiP) and other ER resident proteins (40, 41). BiP is known to associate with unfolded or misfolded proteins and to be retained in the ER (42, 43). Although no information is available about RBP interaction with these proteins during the retinol-deficient state, one can speculate that apo-RBP may be retained in the ER by one of these chaperone proteins under retinol deficiency, and that when retinol is available, correctly folded holo-RBP is transferred to the GC. Conformational changes that are found between apo- and holo-RBP (44) may participate in this phenomenon. Thus, the hypothesized conformational change that occurs when retinol binds to RBP may be the signal for the continuation of intracellular transport (45). This hypothesis coincides with much of the evidence that newly synthesized secretory proteins need to acquire mature tertiary and quaternary structure in order to exit the ER (46).

Colchicine has been shown to cause fibrinogen, very low density lipoprotein, and albumin to accumulate in Golgi and Golgi-derived secretory vesicles in hepatocytes (9–11, 47). In the present study we observed similar effects of colchicine upon RBP secretion. When retinol-deficient rats were pretreated with colchicine, we observed a heavy accumulation of RBP in the Golgi-derived secretory vesicles after retinol repletion (Fig. 8); these data indicate that colchicine inhibits RBP secretion at the transport by secretory vesicles from the GC to the plasmalemma. These observations are in good agreement with available biochemical data (15) on this issue.

A low level of apo-RBP circulates in the plasma of retinol-deficient rats (2, 6, 32). There are two possible sources for this circulating apo-RBP. One is the liver. The avail-

able evidence indicates that retinol deficiency results in a partial inhibition, rather than in a complete block, of RBP secretion by the parenchymal cell (33). Although we cannot support this by our findings, a very small amount of RBP (below detection level of our method) may be secreted by a constitutive pathway (48), which is thought to be a bulk flow mechanism (49), apart from the pathway of regulated secretion. Another possible source of apo-RBP in deficient plasma is represented by the extrahepatic tissues that contain RBP mRNA and presumably synthesize RBP (38). The mechanisms that regulate RBP synthesis and secretion by extrahepatic tissues remain to be explored. In a recent study, Soprano et al. (50) demonstrated that the synthesis and secretion of RBP by the yolk sac placenta of the developing rat are regulated by retinol in a manner similar to that seen in liver. In extrahepatic tissues, as in liver, RBP secretion via constitutive pathway may contribute to plasma apo-RBP in the retinol deficient animal.

There is much evidence that the hepatic perisinusoidal stellate cells (SCs) are the major site of vitamin A storage in liver (51, 52). SCs are located in the perisinusoidal space and contain numerous lipid droplets. Vitamin A, mainly in the form of retinyl esters, has been localized in the lipid droplets of the SCs by fluorescence microscopy (53), by electronmicroscope radioautography (54), and directly by isolation and analysis of the SCs (51, 52, 55, 56). The SCs are enriched in the cellular retinoid-binding proteins, and in the enzymes that synthesize and hydrolyze retinyl esters, when compared to parenchymal cells (51, 52). The mechanism whereby dietary retinol, taken up by liver parenchymal cells, is transferred to SCs for storage is not known. It has been suggested that RBP may be involved in the bidirectional intercellular transfer of retinol between parenchymal and stellate cells (57–59). Our data, however, are not consistent with this hypothesis. Although we have carefully looked for RBP in SCs, we were unable to detect significant RBP staining in these cells. As discussed before, since the reaction product for RBP is observed as an electron-dense deposit, we cannot eliminate the possibility of RBP localization in the periphery of lipid droplets (especially in type I droplets); however, RBP was not seen in organelles associated with the secretory pathway. Our findings agree with published studies that indicate that RBP is not present in significant levels in isolated stellate cells (51, 56), and that RBP mRNA is not synthesized by SCs (56). Taken together, our study and these other published reports would indicate that RBP is neither synthesized nor taken up in substantial quantities by these cells. Thus, if RBP is involved in the bidirectional movement of retinol between parenchymal and SCs in an important way, the transfer of retinol must occur at the surface of the stellate cell. At present no information supporting this hypothesis is available, and it is clear that future research will be necessary to elucidate the mechanism of intercellular transfer of retinol in the liver.

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