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Transfer of retinol-binding protein from HepG2 human hepatoma cells to cocultured rat stellate cells

(perisinusoidal stellate cells/fat-storing cells/vitamin A/retinoids/liver cells)

HARUKI SENOO*, SIGBJØRN SMELAND*, LUCIE MALABA*, TOVE BJERKNES*, ESPEN STANG†, NORBERT ROOS†, TROND BERG*, KAARE R. NORUM*, AND RUNE BLOMHOFF*

*Institute for Nutrition Research, School of Medicine, and †Electronmicroscopical Unit for Biological Sciences, University of Oslo, P.O. Box 1046, 0316 Oslo, Norway

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ABSTRACT **Rat liver stellate cells were cocultured with HepG2 human hepatoma cells, which are known to synthesize and secrete retinol-binding protein (RBP). Transfer of human RBP from HepG2 cells to stellate cells was studied by cryoimmunoelectron microscopy. In stellate cells, human RBP was found on the cell surface and within endosomes. The transfer of human RBP from HepG2 cells to stellate cells was blocked by addition of RBP antibodies to the culture medium. Very little uptake of RBP was observed when fibroblasts were cocultured with HepG2 cells. In a series of experiments, RBP was bound to its putative cell surface receptor at 4°C, and the stellate cells were washed and then incubated at 37°C in order to allow them to internalize a pulse of RBP. About 50% of the RBP was internalized after 6 min of incubation. The RBP-positive vesicles were initially (after 1–2 min) located close to the cell surface and later were found deeper in the cytoplasm. During the first 10 min, RBP was mainly observed in close association with membranes. After 2 hr, however, most RBP was localized in intracellular vesicles at a distance from the vesicular membranes, suggesting that RBP had been released from its receptor. Saturable binding of RBP to liver cells was demonstrated when cells were incubated with ^{125}I-RBP at 4°C and cell-associated radioactivity was determined. The calculated dissociation constant for the specific binding was 12.7 ± 3.2 nM. A binding assay was also developed for determination of solubilized RBP receptor. Solubilized proteins from the nonparenchymal liver cells bound about 30 times more ^{125}I-labeled RBP than did parenchymal cells (based on mass of cell protein). These data suggest that RBP mediates the paracrine transfer of retinol from hepatocytes to perisinusoidal stellate cells in liver and that stellate cells bind and internalize RBP by receptor-mediated endocytosis.**

Vitamin A (retinol) is essential for vision and regulates differentiation and growth of many cell types both during embryonal development and in adult tissues. Thus, many cell types require a continuous supply of retinol. Liver perisinusoidal stellate cells (also called fat-storing cells and lipocytes) play an important role as a main body store of retinol (1, 2). The ability of the stellate cells to control storage (as retinyl esters) and mobilization of retinol ensures that the blood plasma retinol concentration remains close to 2 μM, thus providing ample access of retinol to target cells during periods of low dietary intake (3).

Vitamin A is transported from the intestine to the parenchymal liver cells (hepatocytes) in chylomicrons and their remnants (1, 3). Some years ago we reported that most of the retinol in hepatocytes that are derived from chylomicron remnant retinyl esters are transferred within 2–4 hr to hepatic perisinusoidal stellate cells (4). Chylomicron remnant-derived retinyl esters must be hydrolyzed before retinol is transferred to stellate cells (5, 6). The transfer of retinol from hepatocytes to stellate cells *in vivo* is too rapid to be accounted for by secretion from hepatocytes to the general circulation, followed by uptake into stellate cells.

To get more insight into the mechanism of transfer of retinol between liver cells, we investigated the transfer of retinol to stellate cells in livers perfused *in situ* (5). Antibodies against retinol-binding protein (RBP) effectively reduced the transfer of retinol from hepatocytes to stellate cells (5). In accordance with this model, we reported that stellate cells were active in the uptake of retinol from plasma (7) and we demonstrated by immunoelectron microscopy that RBP was internalized by these cells (8).

To study the mechanism of retinol transfer from parenchymal to stellate cells in more detail, we have now cocultured rat liver stellate cells with a human hepatoma cell line (HepG2). HepG2 cells are known to synthesize and secrete RBP (9). Thus, by using cryoimmunoelectron microscopy and a specific antibody recognizing human RBP, we have examined the uptake of HepG2 cell-derived human RBP by rat liver stellate cells. RBP was also bound to its putative cell surface receptor at 4°C, and stellate cells were washed and then incubated at 37°C in order to allow them to internalize a pulse of RBP.

MATERIALS AND METHODS

Materials. Fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO. Sheep anti-human RBP was obtained from Biogenesis (Bouremouth, U.K.) and rabbit anti-sheep IgG was obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands). Protein A-colloidal gold (5–7 nm in diameter) (10) was kindly donated by Jan W. Slot (Center for Electron Microscopy, School of Medicine, University of Utrecht, The Netherlands). Another protein A-colloidal gold (10 nm in diameter) was prepared and donated by Paul Webster (Yale University). RBP was isolated from human plasma as described previously (11) and iodinated by the Bolton–Hunter or the Enzymobead (Bio-Rad) method (12).

Animals. Male Wistar rats weighing 250–300 g were used. The animals were maintained on an ordinary pellet diet (No. 3155, AREX, Møllesentralen, Norway) that contained about 9.4 μmol of retinoids (50% retinyl acetate and 50% retinyl palmitate) per kilogram. Animals were fasted for 12 hr before the start of an experiment.

Isolation and Cultivation of Stellate Cells, HepG2 Cells, and Skin Fibroblasts. Rat livers were perfused with a collagenase technique (13), and parenchymal and nonparenchymal cells were isolated by differential centrifugation. Pure cultures of

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Abbreviation: RBP, retinol-binding protein.

desmin-positive stellate cells were prepared as described (13). The stellate cells were grown in DMEM containing 20% fetal bovine serum, gentamicin (200 μg/ml), Fungizone (4 μg/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in an atmosphere of 5% CO_2. The medium was changed every second day.

HepG2 cells were obtained from American Type Culture Collection. Skin fibroblasts were prepared from Wistar rats. Fibroblasts and HepG2 cells were grown in medium supplemented with 10% fetal bovine serum, gentamicin (200 μg/ml), Fungizone (4 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and 20 mM Hepes.

Coculture. Transwell porous cell culture inserts (24.5 mm) and suitable six-well cluster dishes (35 mm in diameter) (Costar 3410) were used in the coculture experiments. The bottom of each insert consisted of a polycarbonate membrane with 3-μm pores. HepG2 cells ($1–3 \times 10^5$) were seeded in the upper compartment. Stellate cells or fibroblasts (3×10^5) were seeded in the lower compartment. After separate cultivation for 1–3 days, cells were washed three times in serum-free DMEM and cocultured for 18 hr in the same medium. To block the uptake of human RBP by cultured rat stellate cells, sheep anti-human RBP (diluted 1:60) was present in the medium during coculture.

Fixation of Cell Cultures. After incubation, cell cultures were washed three times with phosphate-buffered saline (PBS: 8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4/0.15 M NaCl, pH 7.2) and fixed with 0.5% glutaraldehyde and 2% formaldehyde in 0.2 M Pipes, pH 7.4/5% sucrose for 30 min at room temperature. After fixation, the cells were collected with a rubber policeman and concentrated by centrifugation. The pellets were washed twice with 0.2 M Pipes, pH 7.4/10% sucrose and then immersed in 0.2 M Pipes, pH 7.4/2.3 M sucrose for 15 min for cryoprotection. The pellets were then cut into small pieces and frozen in liquid nitrogen. In some experiments 0.1 M phosphate buffer (pH 7) was used instead of Pipes in the fixation and washing procedure.

Immunolabeling and Electron Microscopy of Cryosections. Ultrathin cryosections of the specimens were cut on an LKB Ultratome V with a Cryonova unit at approximately −100°C (14, 15). For electron microscopy, sections were put on Formvar carbon-coated copper grids. Indirect immunolabeling was performed at room temperature. The cryosections were first incubated for at least 15 min with 10% newborn calf serum (NCS) to block nonspecific binding of antibody, rinsed with PBS, and incubated for 30 min with sheep anti-human RBP diluted 1:100 in 5% NCS. Sections were then rinsed with PBS, incubated for 30 min with rabbit anti-sheep IgG diluted 1:100 in 5% NCS, rinsed, and, finally incubated for 20 min with protein A-colloidal gold (5–10 nm in diameter). Rinse buffer contained 0.016 M glycine to quench free aldehyde groups (16).

After immunolabeling, cryosections were stained and embedded in a mixture containing 0.3% uranyl acetate and 2% methyl cellulose (17) for 10 min on ice and then observed in a JEOL 100-CX electron microscopy at an accelerating voltage of 80 kV. In control experiments, sections were incubated with sheep anti-human RBP and the same volume of human RBP (0.2 mg/ml), instead of sheep anti-human RBP alone.

Characterization of Sheep Anti-Human RBP Antibody. The sheep antibody against human RBP was characterized by Western blot analysis (18). Rat and human plasma and purified rat and human RBP were subjected to SDS/15% PAGE (19). Proteins in the gel were then transferred to nitrocellulose sheets by electroblotting, and immunoreactive protein was visualized with Vectastain ABC reagents (Vector Laboratories) and 4-chloro-1-naphthol. The antibody was monospecific for human RBP, and it did not crossreact with purified rat RBP or RBP in rat serum. Also, cryosections of liver from control rats showed no positive reaction against sheep anti-human RBP. Hence, positive reaction in the experiments described represents cell-associated human RBP.

Assay for Solubilized Receptor. The incubation mixture (total volume, 0.5 ml) contained cells (at the concentrations given), 4 nM ^{125}I-RBP, 40 mM octyl glucoside, 0.6% bovine serum albumin, 600 mM NaCl, 40 mM $CaCl_2$, and 100 mM $MgCl_2$. Parallel incubations were carried out in the presence of 200-fold excess unlabeled RBP to determine nonspecific binding. The incubation mixture was drawn gently through a pipette and then incubated for 15 min at room temperature. Insoluble material was pelleted by centrifugation at $12,000 \times g$ for 5 min and the supernatant was transferred to new Eppendorf tubes. To this solution was added polyethylene glycol (PEG) 6000 dissolved in PBS, yielding a final concentration of 25% (wt/vol) PEG and 16 mM octyl glucoside (i.e., a concentration that is below the critical micellar concentration of octyl glucoside). The receptor–ligand complexes were pelleted by centrifugation at $12,000 \times g$ for 5 min at 4°C. The pellets were washed twice with ice-cold 25% PEG 6000 solution and counted in a Kontron γ counter.

RESULTS

Uptake of HepG2-Derived RBP by Cocultured Stellate Cells. In the first series of experiments, we tried to mimic the situation in intact liver by coculturing rat liver stellate cells with human HepG2 cells, which are known to synthesize and secrete RBP. Stellate cells were cocultured with HepG2 cells for 18 hr and cryosections from stellate cells were observed by immunoelectron microscopy.

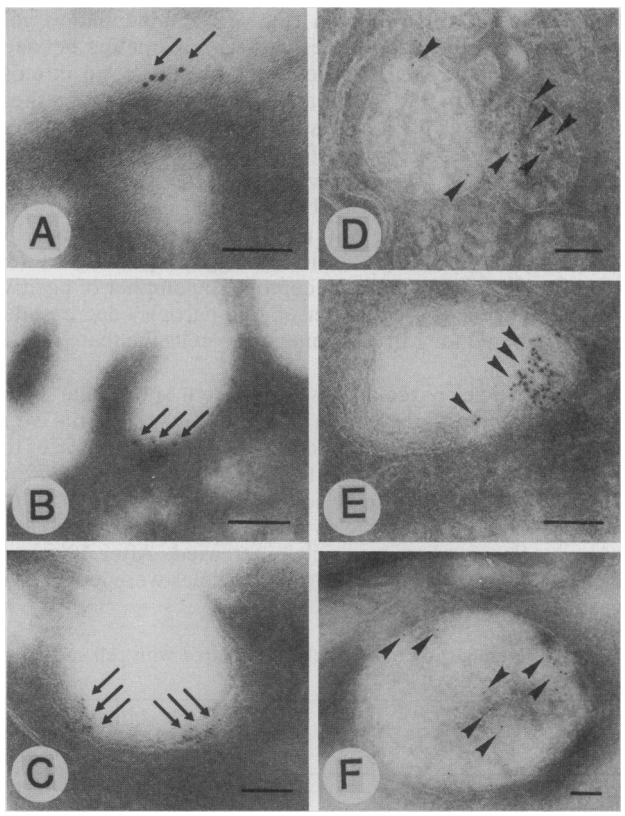

FIG. 1. Immunoelectron micrographs of stellate cells cocultured with HepG2 cells. Indirect immunolabeling was performed as described. Arrows and arrowheads indicate, respectively, gold particles distributed on the cell surface and in the cytoplasm. (Bars = 100 nm.)

Gold particles in stellate cells were distributed on the cell surface (Fig. 1 *A–C*), within pits on the cell surface (Fig. 1 *B* and *C*), and within vesicles in the cytoplasm (Fig. 1 *D–F*). The pits were 100–200 nm in diameter, whereas the vesicles were 450–500 nm in diameter. In some of these vesicles, gold particles were attached to the membranes, while in others, the particles localized within the vesicular lumen. Quantitative data from these experiments are presented in Table 1.

We next tested the ability of antibodies against RBP to inhibit transfer of RBP to stellate cells in the coculture system. Stellate cells were cocultured with HepG2 cells for 18 hr in medium containing anti-human RBP, washed three times, and processed from immunoelectron microscopy. In such sections, much less labeling was observed, suggesting that the antibody blocked the transfer of RBP to stellate cells (Table 1).

To test the selectivity of RBP uptake by stellate cells, we did similar coculture experiments with HepG2 cells and human fibroblasts. Fibroblasts were cocultured with HepG2 cells for 18 hr in control medium, washed three times, and analyzed. Very little labeling by gold particles was detected, both in the endocytic apparatus and in organelles involved in protein synthesis and secretion (Table 1).

Internalization of a Pulse of Cell Surface-Bound RBP by Stellate Cells. At 4°C, no internalization of the plasma membrane will take place. Hence, RBP was bound to receptors on the plasma membrane of cultured stellate cells by incubation of cells with human RBP (50 μg/ml) for 2 hr at 4°C. The cells were washed and then incubated at 37°C for various times. After washing (Fig. 2*A*) and after 1 min of incubation at 37°C (Fig. 2*B*), gold particles were almost exclusively distributed on the cell surface. After 2 min of incubation at 37°C, gold particles were also found in vesicles close to the cell surface (Fig. 2*C*). This inward migration of RBP continued, and after 6 min (Fig. 2*D*) and 10 min (Fig. 2*E*) of incubation gold particles were localized in vesicles with diameters between 200 and 400 nm. The particles were mainly found in close association with the membrane of the vesicles. At these times, almost no gold particles were found on the cell surface.

In cell cultures incubated for 2 hr at 37°C, gold particles were distributed mainly in vesicles located deeper in the cytoplasm, with a diameter between 500 and 600 nm (Fig. 2*F*). At this time point, gold particles were mainly found in the central region of the vesicles, suggesting that very few were bound to the putative receptor. The number of positive vesicles as well as the number of gold particles decreased in cells incubated for 2 hr as compared to cells incubated for 10 min at 37°C.

Gold particles associated with different subcellular structures at each time point in the pulse experiments were counted (Fig. 3). After 1 min of incubation, about 70% of the gold particles were found on the cell surface and only 7% of the particles were within vesicles. With incubation time, the number of the gold particles on the cell surface decreased whereas the number in vesicles increased. After 10 min of incubation at 37°C, almost all gold particles were localized in vesicles.

Table 1. Number of gold particles associated with cell surface and vesicles

	No. of particles	
	Cell surface	Vesicles
Stellate cells	641	438
Stellate cells plus RBP antibodies	70	35
Fibroblasts	41	0

From several experiments similar to that presented in Fig. 1, the numbers of gold particles associated with the cell surface and cytoplasmic vesicles or endosomes were determined over a surface area equaling 0.5 mm^2.

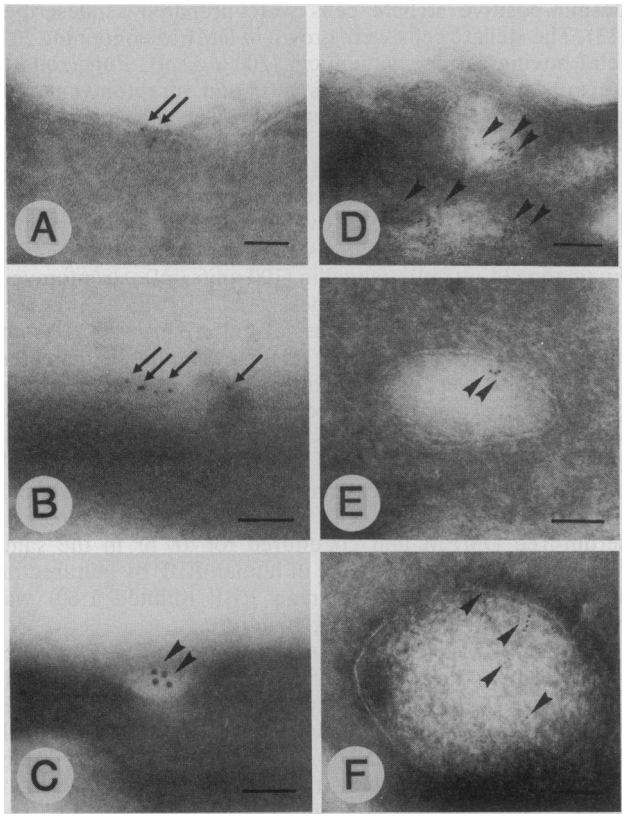

FIG. 2. Immunoelectron micrographs of cultured stellate cells showing subsequent stages in the endocytosis of human RBP. Stellate cells were incubated with human RBP (50 μg/ml) for 2 hr at 4°C, washed (*A*), and then incubated at 37°C for 1 min (*B*), 2 min (*C*), 6 min (*D*), 10 min (*E*), and 120 min (*F*). Arrows and arrowheads indicate, respectively, gold particles distributed on the cell surface and in cytoplasmic vesicles. (Bars = 100 nm.)

Evidence for Saturable Binding of RBP to Liver Cells. Several types of experiments were performed to measure saturable binding of RBP to liver cells. First, liver parenchymal and stellate cells were incubated with various concentrations of ^{125}I-labeled RBP (0.5 nM to 1.0 mM) at 4°C and cell-associated radioactivity was determined. The data showed that the binding of RBP to parenchymal liver cells was saturable (Fig. 4*A*). By nonlinear regression analysis, the calculated dissociation constant (K_d) for the specific binding was 12.7 ± 3.2 nM and the number of binding sites (B_{max}) was 138 ± 19 fmol per 10^6 parenchymal cells.

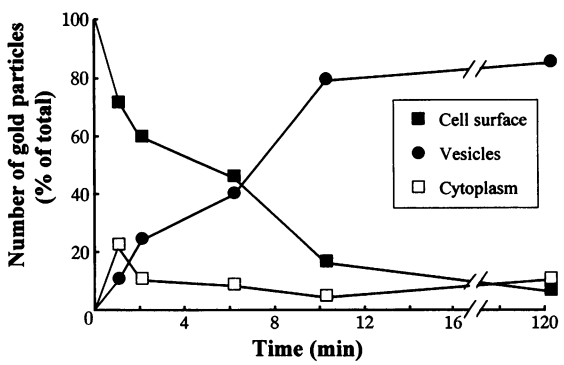

FIG. 3. Counts of gold particles associated with different structures. From several experiments similar to that presented in Fig. 2, gold particles associated with the cell surface (■), cytoplasmic vesicles or endosomes (●), and in the cytoplasm (□) were counted. Two hundred gold particles were counted at each time point.

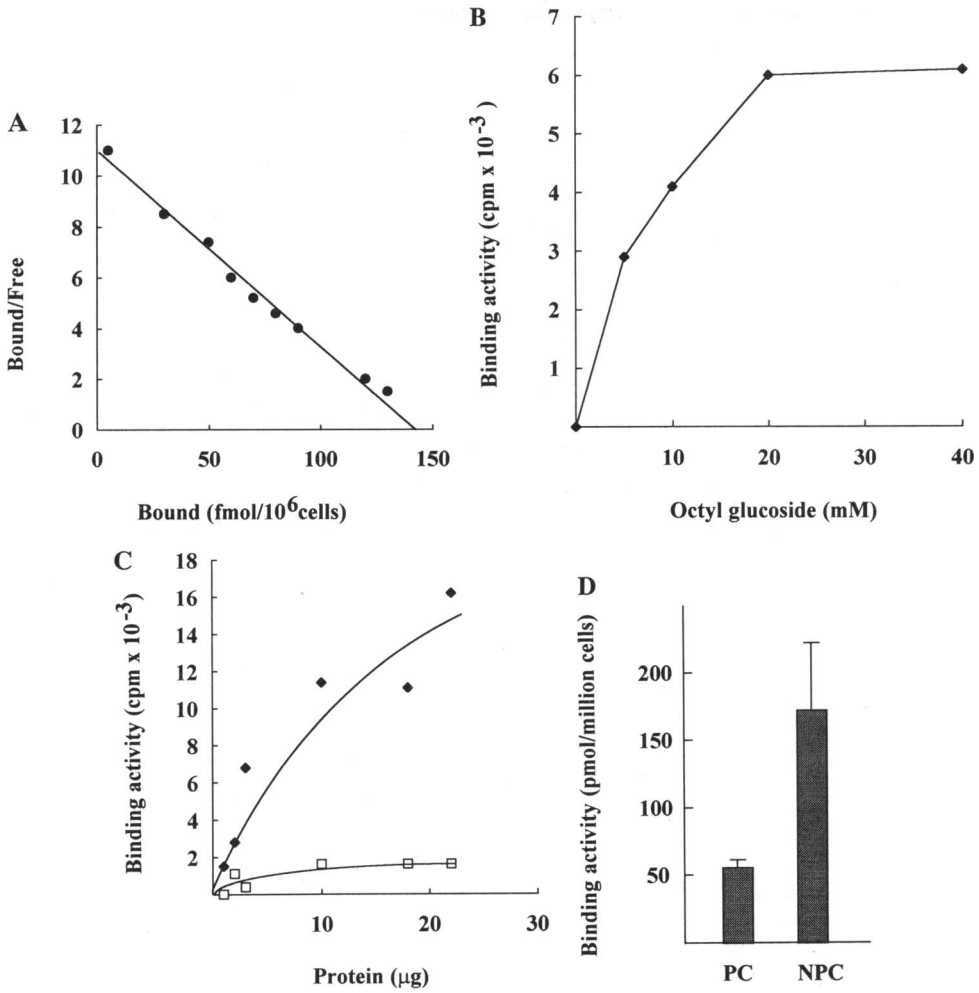

FIG. 4. Saturable binding of RBP to liver cells. (*A*) Binding of RBP to suspended liver parenchymal cells (10^7 per ml) was determined by a centrifugation-through-oil method (12). Cells were incubated at 4°C for 15 min with 0.5 nM ^{125}I-RBP and increasing concentrations (up to 1 μM) of unlabeled RBP. The data were plotted according to Scatchard (20) and the continuous curves were calculated by using the ENZFIT program (Elsevier–Biosoft, U.K.). The values represent means of two to four experiments with duplicate determinations. (*B*) Total liver was solubilized in various concentrations of octyl glucoside and the binding activity was determined by precipitation in 25% PEG. No unbound RBP precipitates at this concentration. (*C*) Binding activity with increasing amounts of solubilized protein from total liver in the absence (◆) or presence (□) of a 200-fold excess of unlabeled RBP. (*D*) Binding activity of solubilized RBP receptor in parenchymal (PC) and nonparenchymal (NPC) liver cells (3 × 10^6 per ml).

It was not possible to perform similar experiments with stellate cells, because of the low number of stellate cells isolated. Stellate cells comprise <5% of the liver cells, and one rat liver yields only about 10 × 10^6 stellate cells compared with 500 × 10^6 parenchymal cells. Therefore, an alternative binding assay was developed.

Total liver was solubilized in various concentrations of octyl glucoside and the binding activity was determined by precipitation in 25% PEG 6000. No unbound RBP precipitated at this concentration of PEG, and free RBP could thus be separated from protein-bound RBP. Optimal solubilization of the receptor occurred at about 20 mM octyl glucoside (Fig. 4*B*). Furthermore, binding increased with the amount of solubilized protein, and 65–90% of the binding of ^{125}I-RBP was blocked when a 200-fold excess of nonradioactive RBP was used as competitor (Fig. 4*C*). Then parenchymal and nonparenchymal cells (3 × 10^6 per ml) were solubilized in 40 mM octyl glucoside and receptor-bound ^{125}I-RBP was determined. Solubilized proteins from the parenchymal and nonparenchymal liver cells bound 67 ± 13 and 176 ± 60 pmol per 10^6 cells (mean ± SD, n = 4), respectively (Fig. 4*D*). Since parenchymal cells contain 10 times as much protein per cell, the data suggest that nonparenchymal cells express about 30 times the binding activity of parenchymal cells (based on mass of cell protein).

DISCUSSION

We have studied the mechanism of the paracrine transfer of retinol from hepatocytes to perisinusoidal stellate cells in liver (4, 5). As a model system we used the human hepatoma cell line HepG2 cocultured with rat liver stellate cells. The HepG2 cells synthesize and secrete RBP, and by using cryoimmunoelectron microscopy and an antibody recognizing human RBP we studied the uptake of HepG2 cell-derived human RBP by rat liver stellate cells.

After coculture, human RBP was localized in various subcellular structures of stellate cells such as the cell surface, pits of the cell surface, and endocytic vesicles. In some of these vesicles, RBP was located close to the membranes, whereas in others, RBP was located within the vesicular lumen. RBP attached to the cell surface or to the vesicular membranes probably represents RBP bound to its putative receptor (21–23). Also, the finding that stellate cells are much more active than fibroblasts in uptake of RBP suggests that a cell surface receptor is involved in RBP uptake by stellate cells.

Several reports suggest that RBP may easily be denatured during the isolation or iodination procedure, thus hampering most binding and uptake studies (12). An important feature of the coculture system used in the present report is therefore that no RBP isolation or iodination was necessary. In this respect it is interesting that the concentration of RBP in the coculture medium was 50 times less (data not shown) than during the uptake experiments where isolated RBP was added to the stellate cell medium. Still, much more RBP was taken up by stellate cells in the coculture system.

In a previous report we showed that retinol was transferred from hepatocytes to stellate cells when rat livers were perfused *in situ* (5). In that study we tested the ability of polyclonal antibodies against RBP to inhibit the transfer of retinol. No transfer of retinol was observed when antibodies were included in the perfusion medium, suggesting that the transfer of retinol was mediated by RBP (5). The ability of an

antibody against RBP to inhibit the transfer of RBP to stellate cells was also tested in the present coculture system. In such experiments, no specific labeling was observed in stellate cells, suggesting that the antibody blocked the transfer of RBP.

In another series of experiments, RBP was bound to its cell surface receptor, and the cells were then allowed to internalize this pulse of RBP. About 50% of the RBP was internalized after 6 min of incubation. The RBP-positive vesicles were initially (after 1–2 min) located near the cell surface. Later, RBP-positive vesicles were observed deeper in the cytoplasm. During the first 10 min, most RBP was associated closely with the plasma membrane or with vesicular membranes. After 120 min of incubation, RBP was localized at a distance from the vesicular membranes.

Although the existence of a cell surface receptor for RBP has been controversial (24, 25), evidence for a saturable plasma membrane receptor that recognizes RBP has been reported in several studies. Heller (21) presented evidence of saturable binding of RBP to retinal pigment epithelial cells. He observed a temperature-dependent and rapid binding of retinol/^{125}I-RBP to the cells. Since he found that unlabeled retinol/RBP could displace the cell-associated retinol/^{125}I-RBP within 3 min, Heller concluded that binding was a surface phenomenon and that retinol was released from RBP and delivered to the cytoplasm from the cell surface. It is also quite clear from the work of Sivaprasadarao and Findlay (22, 23) that cell-free preparations accumulate retinol and that the amount of retinol accumulated is greater than the number of receptors present. Bok and Heller (26) showed that iodinated RBP could be localized on the basal and lateral plasma membrane of retinal pigment epithelial cells after injection into the intact animal, but they saw no evidence for internalization. Also, Shingleton *et al.* (27) found no internalization of RBP by cultured Sertoli cells under conditions in which retinol was being internalized. All of these data suggest that internalization of RBP is not a prerequisite for retinol delivery to cells. On the other hand, in the present and in a previous (8) electron micrographic study we have shown that RBP can be internalized by liver cells. From the data available at present, retinol seems to be released from RBP at the cell surface in some cells and following internalization of the retinol/RBP-complex in other cells.

In summary, this report supports the suggestion that RBP mediates the paracrine transfer of retinol from hepatocytes to perisinusoidal stellate cells in liver and that RBP is internalized by cultured rat liver stellate cells by receptor-mediated endocytosis.

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