# Ligand-dependent Regulation of Intracellular Protein Transport: Effect of Vitamin A on the Secretion of the Retinol-binding Protein

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ABSTRACT As a model of ligand-dependent protein secretion the biosynthesis, intracellular transport, and release of the retinol-binding protein (RBP) were studied in primary cultures of rat hepatocytes pulse-labeled with [$^{35}$S]methionine. After various periods of chase RBP was isolated by immunoprecipitation and identified by SDS PAGE.

Both normal and vitamin A-deficient hepatocytes synthesized RBP. The normal cells secreted the pulse-labeled RBP within 2 h. RBP synthesized by deficient cells was not secreted, and intracellular degradation of the protein appeared to be slow. Deficient cells could be induced to secrete RBP on the addition of retinol to the culture medium. This occurred also after protein synthesis had been blocked by cycloheximide. Since retinol induces the secretion of RBP, accumulated in the endoplasmic reticulum (ER), it seems reasonable to conclude that the transport of RBP from the ER to the Golgi complex is regulated by retinol.

The ultimate localization of newly synthesized membrane and secretory proteins is determined in part by the presence of signal sequences in the nascent proteins (1–3). However, the precise mechanisms by which the cell directs specific proteins to different compartments are yet to be defined.

One way to approach this problem is by genetic analysis. Thus, the isolation of a number of *sec* mutants has provided valuable information on the secretory pathway in yeast (reviewed in reference 4). In higher eukaryotes, this kind of information may be obtained by the use of mutant cell lines that have well defined defects in the processing and intracellular transport of proteins (5). Alternatively, agents that promote the transfer of a newly synthesized protein from one compartment to another may be equally useful. In vivo analyses have suggested that vitamin A may regulate the secretion of retinol-binding protein (RBP) in this manner (6, 7).

RBP, a plasma protein with a molecular weight of 21,000 (7, 8), is responsible for the transport of vitamin A (retinol) from its storage site in the liver to the various vitamin A-dependent tissues (9–12). The secretion of RBP, manufactured by the hepatocytes, is controlled by vitamin A. Thus, vitamin A-deficiency causes the serum concentration of RBP to decrease as a consequence of the hepatic stores of the vitamin being depleted (6, 7). In vivo experiments have suggested that RBP accumulates in vitamin A-deficient hepatocytes, since the secretion but not the synthesis of RBP is impaired in deficient cells (6, 13). The accumulation of RBP appears to be largely confined to the endoplasmic reticulum (12). It has been suggested that the impaired secretion of RBP is resumed when the animals are given retinol and that this vitamin A-induced secretion is independent of protein synthesis (7, 14).

These in vivo data suggest that the intracellular transport and secretion of RBP are precisely controlled by vitamin A-dependent mechanism(s). Thus, the intracellular transport of RBP may lend itself to detailed mechanistic analyses provided appropriate in vitro systems are available. We have established an in vitro tissue culture system which faithfully seems to reproduce the in vivo situation. In this communication we provide direct evidence that the secretion of RBP in primary cultures of rat hepatocytes is strictly regulated by retinol.

## MATERIALS AND METHODS

*Rat Hepatocytes:* Weanling male Sprague-Dawley rats were obtained from Anticimex (Stockholm, Sweden). Retinol-deficiency was induced as described (15). The deficient animals were sacrificed during the retinoic acid-free phase of the feeding cycle (see reference 15).

Hepatocytes from deficient and normal rats were prepared by a collagenase perfusion technique as described earlier (16). Hepatocytes were plated into 60-mm Falcon tissue culture dishes (Falcon Labware, Oxnard, CA) and allowed to settle for 1 h at 37°C. To each dish, $10 \times 10^6$ cells in 5 ml of Buffer 3 of reference 16 were added. The dishes were precoated with 20 μg of bovine fibronectin (17).

*Labeling of Cells with [$^{35}$S]Methionine:* Cell monolayers were washed three times with a balanced salt solution and then preincubated for 1 h with the labeling medium (methionine-free Ham's F-10 medium supplemented with 6 mM glutamine). [$^{35}$S]Methionine (New England Nuclear, Boston, MA; specific activity 1,000 μCi/mmol) was then added to each dish in 3 ml of fresh labeling medium. Typically, 100 μCi was used to label $10 \times 10^6$ cells. After labeling, the cells were maintained in chase medium (Ham's F-10 medium to which had been added 50 times the normal amount of unlabeled methionine). Retinol, when present, was added in ethanol solution to a final ethanol concentration of 0.5% in the incubation medium.

To avoid possible interference of serum RBP and vitamin A with the experiments, we included no serum in the labeling and chase media. The omission of

serum did not markedly affect the viability of the cells, when incubated for periods of a few hours. Neither the adherence to the dishes nor the incorporation of [$^{35}$S]methionine into protein was substantially reduced.

*Solubilization and Immunoprecipitation:* At the end of each incubation period the medium was removed and the cell monolayer was solubilized in 3 ml of ice-cold 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, 1% Triton X-100, 0.02% $NaN_3$, and 6 mM phenyl methyl sulfonyl fluoride (PMSF). The incubation medium was chilled on ice and centrifuged briefly to remove occasional nonadherent cells. Triton X-100 and PMSF were then added to final concentrations of 1% and 6 mM, respectively. After 10 min on ice the solubilisate and the medium were separately centrifuged for 20 min at 2,000 *g*. The resulting supernatants were each incubated overnight with 25 μl of normal rabbit serum. Material sticking nonspecifically to IgG was removed by the addition of formalin-fixed *Staphylococcus aureus* bacteria (18). The supernatants recovered were incubated for 4 h with 5 μl of a rabbit antiserum against rat RBP (19). Immune complexes were isolated and analyzed by SDS PAGE as detailed in reference 20.

The amount of labeled RBP in the immunoprecipitates was determined by autoradiography and densitometric scanning of the 21,000-dalton band.

*Purification and Cell-free Translation of mRNA:* Membrane-bound mRNA was purified from rat liver microsomes. The microsomes were isolated essentially as described (21), but in the presence of 100 μg/ml cycloheximide and 2 $A_{280}$ units/l of the human placental ribonuclease inhibitor (22). Extraction of the mRNA from the microsomes, sucrose gradient fractionation of this mRNA, and in vitro translation in the presence of dog pancreas microsomes were carried out as detailed elsewhere (20).

## RESULTS

### *Synthesis of RBP in Rat Hepatocytes*

Freshly prepared rat hepatocytes were tested for incorporation of [$^{35}$S]methionine into protein. The cells exhibited a linear rate of incorporation of radioactivity into TCA-precipitable material, following an initial lag period of about 8 min. To analyze whether the protein synthesized by the hepatocytes included RBP, we subjected the labeled proteins to immunoprecipitation. The cells synthesized a 21,000-dalton polypeptide reactive with the antiserum (Fig. 1). The same protein was found also in the incubation medium. Thus, rat RBP, with a mol wt of 21,000 (7), appeared to be released from the hepatocytes by a secretory mechanism.

### *The Effect of the Vitamin A Status on the RBP Secretion*

The RBP secretion in normal and vitamin A-deficient hepatocytes was examined by pulse-chase experiments. Cells isolated from normal and deficient rats were labeled with [$^{35}$S]methionine for 30 min and subsequently cultured for various periods of time in an excess of unlabeled methionine. RBP was isolated by immunoprecipitation both from the solubilized cells and from the medium. Following SDS PAGE the radioactivity associated with RBP was quantitated. Fig. 2 shows that both normal and deficient cells synthesized the protein but only the normal cells secreted significant amounts of RBP into the medium. No degradation of the labeled RBP that accumulated in the vitamin A-deficient cells was observed during the course of these experiments.


FIGURE 1 Biosynthesis and secretion of RBP by rat hepatocytes. The cells were incubated for 4 h with 0.5 mCi of [$^{35}$S]methionine. The solubilisate (lane *A*) and the incubation medium (lane *B*) were immunoprecipitated with an antiserum against rat RBP. As a control, the solubilisate (lane *C*) and the medium (lane *D*) were immunoprecipitated with a normal rabbit serum. Immunoprecipitates were analyzed by SDS PAGE. The numbers denote the molecular weights in kilodaltons of marker proteins run in parallel.

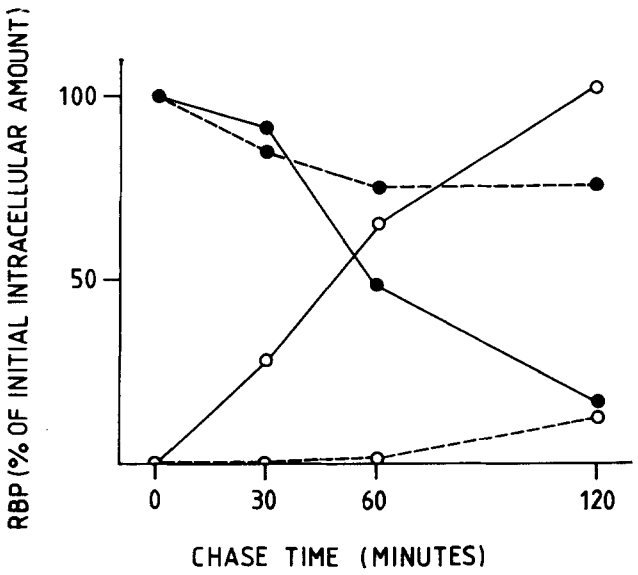


FIGURE 2 Pulse-chase experiment with normal and vitamin A-deficient hepatocytes. Each dish was incubated with 0.5 mCi of [$^{35}$S]-methionine for 30 min. The chase periods lasted for 0, 30, 60, and 120 min, respectively. Intracellular and secreted RBP was immunoprecipitated and the amount of RBP in each sample was estimated by SDS PAGE, autoradiography and densitometric scanning of the 21,000-dalton band. (● —— ●) Intracellular RBP, normal cells. (○ —— ○) RBP in the chase medium, normal cells. (● --- ●) Intracellular RBP, deficient cells. (○- - -○) RBP in the chase medium, deficient cells.

### *Effects of Retinol and Cycloheximide on the RBP Secretion*

Vitamin A-deficient hepatocytes were pulse-labeled with [$^{35}$S]methionine and then incubated with various concentrations of retinol. Immunoreactive RBP in the cells and in the medium was isolated and subjected to SDS PAGE. A dose-dependent release of newly synthesized RBP was induced by retinol, with a lower limit for detectable RBP secretion at a retinol concentration of 50 nM (data not shown). To examine whether the induction of RBP secretion by vitamin A was dependent on continued protein synthesis, we labeled hepatocytes from vitamin A-deficient rats with [$^{35}$S]methionine. Protein synthesis was then interrupted by the addition of cycloheximide. The cells were subsequently incubated with retinol in order to induce RBP secretion. After 2 h the presence of labeled RBP in the cells and in the medium was measured. Fig. 3 shows that the retinol-induced secretion of RBP from vitamin A-deficient hepatocytes occurred also in cells treated with cycloheximide. The data suggested that the secretion of RBP was of similar magnitude with or without cycloheximide treatment (not shown).

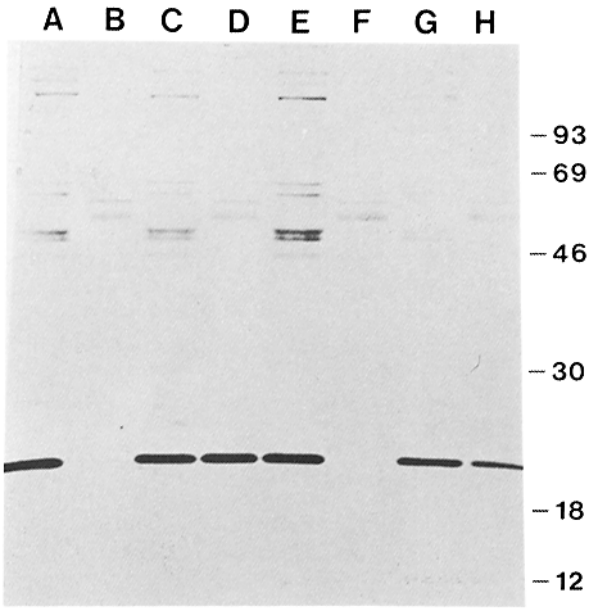


FIGURE 3 Effect of cycloheximide on the RBP secretion in vitamin A-deficient hepatocytes. The cells were incubated with [$^{35}$S]methionine (120 μCi/dish) for 60 min, followed by a chase period of 10 min during which some dishes received cycloheximide (final concentration 20 μM). Subsequently, fresh chase medium was added to the dishes and the cells were incubated for 2 h in the presence or in the absence of 5 μM retinol. Those cells that had been preincubated with 20 μM cycloheximide also received cycloheximide during this second chase period. At the end of the second chase, RBP was immunoprecipitated from the cells (lanes *A, C, E,* and *G*) and the chase medium (lanes *B, D, F,* and *H*) and analyzed by SDS PAGE. Lanes *A* and *B*: chase in the absence of cycloheximide and retinol; lanes *C* and *D*: chase in the presence of retinol but not of cycloheximide; lanes *E* and *F*: chase in the presence of cycloheximide but not of retinol; lanes *G* and *H*: chase in the presence of cycloheximide and retinol. The numbers denote the molecular weights in kilodaltons of marker proteins run in parallel.

### *Cell-free Translation of Liver mRNA Coding for RBP*

Membrane-bound rat liver mRNA was isolated, size fractionated by sucrose gradient centrifugation, and subjected to cell-free translation. The mRNA coding for RBP was found in the 13S region. Translation of the mRNA for RBP in the absence of dog pancreas microsomes produced a 24,000-dalton polypeptide. In the presence of microsomes the mature 21,000-dalton polypeptide was obtained (data not shown), in agreement with previous data (23).

## DISCUSSION

Previous in vivo experiments have suggested that newly synthesized RBP is released from the liver only when adequate amounts of vitamin A are available (7, 14). An effect of vitamin A on the release of RBP has also been demonstrated in a rat hepatoma cell line (24). In this study we have shown that the hepatic secretion of RBP may be faithfully reproduced in vitro using primary rat hepatocyte cultures. Thus, RBP is synthesized by both normal and vitamin A-deficient hepatocytes in vitro, and like the situation in vivo, only the normal hepatocytes secrete the protein. However, if the vitamin A-deficient hepatocytes are cultured in the presence of retinol, these cells will also secrete RBP. These observations are fully concordant with data demonstrating that vitamin A-deficient rats have a very low plasma level of RBP, which upon administration of retinol to the animals is promptly elevated (7, 14). Moreover, the in vitro cultured hepatocytes of deficient rats respond to retinol by secreting RBP with kinetics very similar to those noted in vivo (7, 14).

A crucial observation as regards the RBP secretion is that the release of RBP by retinol-fed hepatocytes of deficient rats takes place also when protein synthesis has been abrogated. This has previously been suggested on the basis of in vivo data (7, 14). However, vitamin A-deficient animals receiving protein synthesis inhibitors are affected generally by the drugs (7, 14), which made the observations open to criticism. The present in vitro system avoids this experimental ambiguity and it can be concluded that retinol induces the secretion of prefabricated RBP. Thus, the vitamin A-dependent regulation of the RBP secretion seems to occur posttranslationally.

RBP synthesized in vitamin A-deficient hepatocytes does not seem to exhibit a very high turnover, as evidenced from the pulse-chase experiments. This may explain the finding that RBP occurs in elevated concentrations in livers of vitamin A-deficient animals (6, 12). Although the level of RBP is somewhat increased in the Golgi fraction, the endoplasmic reticulum seems to retain most of the intracellular RBP in vitamin A-deficient hepatocytes (12). Thus, it seems reasonable to conclude that retinol is the trigger of the RBP secretion and that the ligand exerts its influence either at the level of exit from the endoplasmic reticulum or at the level of entry into the Golgi complex.

The precise mechanism of the retinol-dependent secretion of RBP remains to be elucidated. At least three possibilities can be envisaged. First, retinol may affect the intracellular transport of proteins in general. This possibility seems remote, since the transport of albumin, ceruloplasmin, class I transplantation antigens (12), and transferrin (unpublished observation) seems to occur normally in vitamin A-deficient hepatocytes. Second, it is conceivable that retinol exerts its effect on the secretion of RBP indirectly, by acting as a cofactor of an enzyme causing posttranslational modification of RBP necessary for its secretion. An example of this kind of an effect is found in the secretion of rat prothrombin, which will not be secreted unless a vitamin K-dependent modification of the molecule has occurred (25). However, no evidence of such a posttranslational modification of RBP was obtained in the present investigation. Third, the secretion of RBP may be dependent on the conformation of the protein. Thus, the binding of retinol to RBP may induce a conformational change that exposes a previously buried "signal portion" of the molecule (see reference 3). Alternatively, the apo-protein may be bound to a receptor in the endoplasmic reticulum which has no affinity for the holoprotein. Whatever the molecular mechanism is, primary rat hepatocyte cultures and cell-free translation of mRNA coding for RBP in the presence of microsomes may afford excellent and complementary model systems.

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