Receptor-mediated endocytosis of retinol-binding protein by liver parenchymal cells: interference by radioactive iodination

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Retinol-binding protein (RBP) was iodinated directly by radioiodine substitution on the tyrosyl residues by the sodium hypochlorite (NaOCl) or the Enzymobead (EB) methods, or indirectly by linkage of ^{125}I -tyramine–cellobiose (TC) or ^{125}I -*N*-succinimidyl-3-(4-hydroxyphenyl)propionic acid ester (SHPP) adduct on to free amino residues of RBP. Binding, uptake and degradation of iodinated RBP were studied in isolated rat and rabbit liver parenchymal cells. The amount of ligand bound to cells at 4 °C was dependent on the type of labelling, in that the ^{125}I -TC ligand was bound to a lesser extent than NaClO-labelled ^{125}I -RBP, EB-labelled ^{125}I -RBP and ^{125}I -SHPP-RBP. At 37 °C, the ^{125}I -SHPP-RBP and the EB-labelled ^{125}I -RBP became cell-associated more rapidly than the other two ligands. The higher cell association at 37 °C than at 4 °C suggests that internalization of the ligand occurred at the higher temperature. The degradation of the ligands was also different. The EB-labelled ^{125}I -RBP, the ^{125}I -TC-RBP and the ^{125}I -SHPP-RBP showed an apparent lag

phase before a steady increase in acid-soluble radioactivity was observed. Much less of EB-labelled ^{125}I -RBP and ^{125}I -TC-RBP were degraded (about 6 %) than of the other two ligands (about 16 %) after 120 min. About 50 % of the acid-soluble radioactivity in these experiments could be accounted for by degradation in the medium, suggesting that about half of the degradation observed was intracellular. The present study therefore shows that the different labelling techniques yield varying estimates of the cellular handling of RBP. In addition, a rapid release of RBP was observed in experiments where cells were pulsed with radioactive RBP at 4 °C, washed and incubated further at 37 °C. Between 50 % and 70 % was released after 5 min of incubation. By increasing the temperature during the pulse to 37 °C, or by lowering the temperature during the chase to 4 °C, much less RBP was released from the cells. These data suggest that the release process represents recycling of internalized ligand from an early endosome.

INTRODUCTION

Dietary vitamin A is transported as retinyl esters from the intestine to the liver parenchymal cells in association with chylomicrons [1,2]. After hydrolysis of the retinyl esters to retinol in liver parenchymal cells, retinol is subsequently transferred to liver perisinusoidal stellate cells, re-esterified and stored. Mobilization of the vitamin from the stellate cells involves hydrolysis of the retinyl esters to retinol. In this form, vitamin A is transported in the plasma bound to a specific transport protein, retinol-binding protein (RBP) [3]. RBP in turn normally circulates in plasma as a 1:1 molar complex with transthyretin. This association is thought to prevent renal glomerular filtration of the uncomplexed RBP [4].

Tracer kinetic studies [5] indicate that most of the plasma retinol that leaves the general circulation is recycled rather than being irreversibly utilized in tissues. Recent studies [6] have demonstrated that the liver is responsible for about 20 % of the output of retinol from the plasma retinol–RBP pool. The detailed mechanisms by which RBP-bound retinol is taken up by liver cells is unknown.

The labelling methods for RBP have included immunolabelling *in situ* [7], use of fluorescent dyes [8] and labelling with radioactive isotopes, as in [^3H]retinol [9–12] or radioactive iodine [9–12]. RBP has been labelled with ^{125}I by the chloramine-T [13], the sodium hypochlorite (NaOCl) [14] and the Enzymobeads (EB) [25] procedures for this purpose. By these methods, ^{125}I is directly substituted into the tyrosyl residue, and the resulting radio-labelled catabolic products rapidly escape the cells as iodine or iodotyrosine. Consequently the label recovered in the cells at any

time bears no direct relationship to the ongoing rate of catabolism by those cells. On the other hand, iodinated tyramine–cellobiose (TC) linked on to free amino residues (mostly lysine) of proteins is trapped intracellularly on degradation of the protein.

Several aspects should be considered before selecting the method for protein iodination. First, in proteins with the tyrosyl or lysyl residues as part of the binding domain, the label might interfere with binding and competition studies. Second, some proteins, e.g. parathyroid hormone and calcitonin, are regularly altered during iodination [15]. Exposure of the proteins to chloramine-T, a strong oxidizing agent and subsequent exposure to sodium metabisulphite, a reducing agent, may result in disruption of the protein structure, rendering them unsuitable for use. In order to circumvent these undesirable consequences, milder oxidation methods such as the Enzymobeads procedure have been developed. Also, a method of labelling proteins with ^{125}I was developed [16], in which the protein itself is not exposed to the ^{125}I solution or to the reagents used in the iodination reaction. In this procedure, the protein is treated under mild conditions with the *N*-hydroxysuccinimide ester of 3-(4-hydroxyphenyl)propionic acid (SHPP) that has previously been radioiodinated and solvent-extracted to separate it from the iodination reaction mixture and products. Hydroxysuccinimide esters are known to form amides on reaction with free amino groups of proteins or peptides [17]. Protein hormones labelled by this method have been found to retain their efficacy and ability to react with specific antisera.

In the present work, we have labelled human RBP with ^{125}I , using four different methods. We have used these ligands to study binding, uptake, release and degradation of human RBP in

Abbreviations used: RBP, retinol-binding protein; TC, tyramine–cellobiose; EB, Enzymobeads; SHPP, *N*-succinimidyl 3-(4-hydroxyphenyl)propionic acid ester.

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isolated liver parenchymal cells in an attempt to elucidate the mechanism of uptake of the retinol-RBP complex from the plasma by the liver.

MATERIALS AND METHODS

Chemicals

Na¹²⁵I was purchased from DuPont-New England Nuclear (Boston, MA, U.S.A.). Collagenase and SHPP (commonly known as the Bolton-Hunter reagent) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) and Pierce Europe B.V. (Oud-Beijerland, The Netherlands), respectively. Enzymo-beads were purchased from Bio-Rad Laboratories. Human RBP was purified by a modification of the procedure described by Vahlquist et al. [18]. The RBP preparation was saturated with 70–80% retinol.

Radiolodination of RBP

Human RBP was iodinated with Na¹²⁵I by using four methods:

(a) by direct substitution of the radiolabelled iodine on to tyrosyl residues, by the NaOCl method [14];

(b) indirectly, by the covalent attachment of Iodogen-iodinated TC adduct [19], on to free amino groups (mostly lysine residues) of the RBP;

(c) indirectly, by amide-bond linkage of ¹²⁵I-labelled SHPP to free amino groups (mostly lysine residues) in the RBP molecule [16];

(d) by direct substitution of the radiolabelled iodine on to tyrosyl residues, by using the EB method as described in the instructions of the manufacturer.

Isolation of hepatocytes

Parenchymal liver cells (hepatocytes) were prepared from male Wistar rats (150–300 g) and Chinchilla rabbits (2.5 kg) by a modified [20] collagenase-perfusion technique [21]. The hepatocytes were then washed three times in ice-cold minimal salt solution, pH 7.5, containing 1% (w/v) BSA [20] (incubation buffer E), and finally resuspended in the same buffer. The viability of the hepatocytes, as determined by the Trypan Blue exclusion test, was over 90%.

Binding and uptake experiments

Binding and uptake assays were performed in a shaking water bath at 4 °C and 37 °C, respectively. The lower temperature was chosen since no internalization of plasma membrane occurs at 4 °C, thus making it possible to measure binding of ligands to cell-surface receptors. Binding at each selected time point was determined by transferring a sample (0.5 ml) of the incubation mixture to 10 vol. (5 ml) of ice-cold incubation medium, and centrifuging for 2 min at 16 g. Then the cells were resuspended followed by two further washes by centrifugation to remove unbound radioactivity. The bound radioactivity was calculated as a percentage of total radioactivity in the incubation mixture.

Release and pulse-chase experiments

Hepatocytes were incubated at 4 °C or 37 °C for various time periods in the presence of radioiodinated RBP, followed by three washes, each in 10 vol. of ice-cold incubation medium, to remove

unbound ligand. The cells were resuspended in the original volume of medium and further incubated at 4 °C or 37 °C without extracellular ligand. Duplicate samples of incubation mixture were taken at various time points as described in the experiments above, washed once and thereafter counted for cell-associated radioactivity.

Cell-association studies using centrifugation through oil to separate cells from medium

Cells were incubated with radioactive RBP. At different times of incubation, 100 µl of the incubation mixture was transferred to pre-cooled polypropylene tubes containing 300 µl of dibutyl phthalate/dinonyl phthalate (4:1, v/v). Bound ligand was separated from unbound by centrifugation for 30 s at 2000 g in a Beckman 152 Microfuge. The bottom of the tube containing the cells and bound ligand was cut off and counted for radioactivity.

Degradation of radiolodinated RBP

Degradation of ¹²⁵I-labelled RBP and the ¹²⁵I-SHPP-RBP was determined by precipitation of a 0.25 ml sample of the reaction mixture with an equal volume of 4% (w/v) phosphotungstic acid in 4 M HCl. After standing for at least 20 min in the cold, the precipitate was sedimented by centrifugation. A portion (0.25 ml) of the supernatant was counted for acid-soluble radioactivity. With ¹²⁵I-TC-labelled RBP, 20% (w/v) trichloroacetic acid was used, because much of the free ¹²⁵I-TC was precipitated by 4% phosphotungstic acid. In both cases, the degree of degradation was determined as acid-soluble radioactivity as a percentage of the total radioactivity in the reaction mixture.

RESULTS

Comparison of three labelling techniques

Figure 1 shows comparative data for binding at 4 °C, cell association at 37 °C and degradation at 37 °C of 5 nM RBP labelled by three different techniques. The binding at 4 °C of NaClO-labelled ¹²⁵I-RBP and ¹²⁵I-SHPP-RBP was more or less the same, whereas the binding of ¹²⁵I-TC-RBP was only about one-third of that seen with the other two ligands.

Differences were also observed in the cell association at 37 °C. The cell-associated ¹²⁵I-SHPP-RBP increased most rapidly, with 2.6% of the radioactivity being cell-associated within 5 min of incubation, compared with 1.1% for the NaClO-labelled ¹²⁵I-RBP and 1.0% for the ¹²⁵I-TC-RBP. After 120 min the ¹²⁵I-TC-RBP had accumulated the most (7.2%). Whereas there was no increase in cell-associated NaClO-labelled ¹²⁵I-RBP from 4 °C to 37 °C, there was an increase with ¹²⁵I-TC-RBP and NaClO-labelled ¹²⁵I-SHPP-RBP, suggesting that the degradation products from the last two ligands accumulated in the cells.

Total degradation of the ligands was different. The ¹²⁵I-TC-RBP and ¹²⁵I-SHPP-RBP, unlike labelled ¹²⁵I-RBP, showed an apparent lag phase before a steady increase in acid-soluble radioactivity was observed. Much less ¹²⁵I-TC-RBP was degraded (about 6%) than the other two ligands (about 16%) after 120 min. About 50% of the acid-soluble radioactivity in these experiments could be accounted for by degradation in the medium (as shown in experiments with conditioned medium).

Release and degradation of radioiodinated RBP

When cells were allowed to bind 5 nM radioactive RBP for 60 min at 4 °C, washed to remove unbound ligand and incubated at 37 °C without extracellular ligand, the bound ligand was

Figure 1 Binding (a), uptake (b) and degradation (c) of NaClO-labelled ^{125}I -RBP (●), ^{125}I -TC-RBP (△) and ^{125}I -SHPP-RBP (○) (5 nM)

Data from comparative experiments were normalized against TC-RBP, as this ligand was used in parallel incubations using the same cell preparation in both sets of experiments. The values represent means of three separate experiments. Fractional Standard Deviation (FSD) was less than 9% in this experiment.

rapidly released into the medium. Figure 2 shows comparative data for the three ligands. Within 5 min of incubation, about 50–70% of the bound ligand had been released into the medium. The release process was almost complete after 15 min. After 90 min, about 20% of the NaClO-labelled ^{125}I -RBP and ^{125}I -SHPP-RBP and about 50% of the ^{125}I -TC-RBP remained cell-associated.

Total acid-soluble radioactivity values differed greatly for the three ligands. After 90 min, the NaClO-labelled ^{125}I -RBP was degraded the most (49%), followed by ^{125}I -TC-RBP (23.5%), with ^{125}I -SHPP-RBP being the least degraded (13.7%).

Release of SHPP-RBP at 4 °C and 37 °C

The effects of pulse and chase temperatures on cell-associated ^{125}I -SHPP-RBP are summarized in Figure 3. When the cells were given a pulse at 37 °C, a greater percentage of ligand was cell-associated after a chase at 4 °C than at 37 °C. Similarly, after a pulse at 4 °C, a greater percentage was cell-associated in those cells which had been chased at 4 °C than in those at 37 °C. These data suggest that the ligand is internalized during the 37 °C pulse period, and that recycling of internalized RBP is decreased at low temperature. Similar results were obtained when the NaClO-labelled ^{125}I -RBP was used (results not shown).

Figure 2 Release of NaClO-labelled ^{125}I -RBP (●), ^{125}I -TC-RBP (△) and ^{125}I -SHPP-RBP (○)

Radioactive RBP was added to a rat liver parenchymal-cell suspension to a final concentration of 5 nM and incubated at 4 °C for 60 min. The cells were washed three times to remove unbound ligand, resuspended in the original volume of incubation buffer E and incubated at 37 °C without extracellular ligand. Duplicate 0.5 ml samples were taken for determination of cell-associated radioactivity. The values represent means of three separate experiments. Fractional Standard Deviation (FSD) was less than 6% in this experiment.

Figure 3 Release of ^{125}I -SHPP-RBP

Radioactive RBP was added to a rat liver parenchymal-cell suspension to a final concentration of about 5 nM and incubated at 4 °C (△, ▲) or 37 °C (○, ●) for 60 min. The cells were washed three times to remove unbound ligand, resuspended in the original volume of incubation buffer and incubated at 4 °C (●, ▲) or 37 °C (○, △) without extracellular ligand. Duplicate 0.5 ml samples were taken for determination of cell-associated radioactivity. Values represent means of three separate experiments. Fractional Standard Deviation (FSD) was less than 8% in this experiment.

Comparison of two methods for measuring cell-associated radioactivity

In view of the fast release of the ^{125}I -SHPP-RBP observed, cell-association assays at both 4 °C and 37 °C were repeated by a phthalate/oil separation method [22]. One advantage with this method is that the medium can be separated very rapidly from the cells. These results were compared with those of a duplicate reaction mixture treated in the conventional way (i.e. repeated centrifugations).

The cell-association data obtained from the oil method at 4 °C and 37 °C were about 25–50% higher than with the conventional method, with the greatest difference during the first 15 min (Figure 4). Hence this method offers a faster alternative for

Figure 4 Binding at 4 °C (▲, ●) and uptake at 37 °C (△, ○) of ^{125}I -SHPP-RBP (5 nM) by rat liver parenchymal cells

Cells were separated from the medium by the 'centrifugation-through-oil' method (△, ▲) or by repeated centrifugations (○, ●) as described in the Materials and methods section. The values represent means of three separate experiments. Fractional Standard Deviation (FSD) was less than 5% in this experiment.

Figure 5 Binding of EB-labelled ^{125}I -RBP to suspend rabbit liver parenchymal cells (10^7 cells/ml)

Binding was determined by the centrifugation-through-oil method, as described in the Materials and methods section. Cells were incubated at 4 °C with 0.5 nM EB-labelled ^{125}I -RBP and increasing concentrations of unlabelled RBP. The cell-associated radioactivity was determined after 15 min of incubation. Inset: the same data were plotted in accordance with Scatchard, and the continuous curves were calculated by using the ENZFIT program. The values represent means of 2–4 separate experiments with duplicate determinations. Fractional Standard Deviation (FSD) was less than 8% in this experiment.

separation of unbound ligand before any dissociation of the bound ligand has occurred. Consequently, the oil method was used in the subsequent experiments.

Comparison of the EB- and SHPP-labelled RBP

In the next series of experiments EB-labelled ^{125}I -RBP (5 nM) and ^{125}I -SHPP-RBP (5 nM) were compared in binding, uptake

and degradation studies with rabbit parenchymal cells. Comparable amounts of EB-labelled ^{125}I -RBP and ^{125}I -SHPP-RBP were bound to the cells at 4 °C, and taken up by the cells at 37 °C. The degradation of the ligands was also measured at 37 °C. Both EB-labelled ^{125}I -RBP and the ^{125}I -SHPP-RBP showed a lag phase (5–15 min) before a steady increase in acid-soluble radioactivity was observed. However, much less EB-labelled ^{125}I -RBP was degraded (about 6%) than the other ligand (about 16%) after 120 min. The same results were obtained by using rat parenchymal cells (results not shown).

Saturation of RBP binding to parenchymal cells

Cell-association assays with increasing concentrations (0.5 nM–1.0 μM) of iodinated RBP by rat and rabbit parenchymal cells were performed at 4 °C. When NaClO-labelled ^{125}I -RBP, ^{125}I -SHPP-RBP and ^{125}I -TC-RBP were used, the binding at 4 °C was saturable at concentrations up to about 30 nM. However, we noted that at higher concentrations the percentage of cell-associated radioactivity increased (results not shown). The binding of EB-labelled ^{125}I -RBP was, however, saturable with concentrations up to 1.0 μM (Figure 5). These binding data were plotted in accordance with Scatchard (Figure 5). By using non-linear regression analysis (ENZFIT), the calculated dissociation constant (K_d) for the specific binding was 12.7 ± 3.2 nM and B_{max} was 138 ± 19 fmol/ 10^6 cells. When one high- and one low-affinity binding site are assumed, the calculated parameters were as follows: $K_{d1} = 2.4 \pm 1.6$ nM, $K_{d2} = 87.3 \pm 19.9$ nM, $B_{\text{max},1} = 38 \pm 14$ fmol/ 10^6 cells, $B_{\text{max},2} = 399 \pm 23$ fmol/ 10^6 cells.

DISCUSSION

Evidence from experiments *in vivo* [7,23,24] suggests that both parenchymal and stellate cells in the liver contain receptors for RBP, that RBP is internalized within these cells, and part of that which is internalized is degraded. In the present work, we have studied the binding *in vitro*, uptake, release and degradation of RBP in liver parenchymal cells. RBP was labelled directly by radioiodine substitution on the tyrosyl residues of the RBP and indirectly by linkage of ^{125}I -TC or ^{125}I -SHPP adduct on to free amino residues of RBP.

At 37 °C, the ^{125}I -SHPP-RBP and the EB-labelled ^{125}I -RBP became cell-associated more rapidly than the other two ligands. The amount of ligand bound at 4 °C was also dependent on the type of labelling, the ^{125}I -TC-RBP being bound to a lesser extent than the other ligands. The large difference between cell-associated radioactivity at 4 °C and at 37 °C suggest that internalization of the ligand occurred at the higher temperature. This effect was most evident with ^{125}I -TC-RBP, probably owing to the fact that degradation products from this ligand were trapped most efficiently within the cells. On the other hand, the ^{125}I -RBP whose degradation products rapidly escaped from the cells did not show this effect.

The rapid release of RBP from the cells observed in the present study could be due to a fast dissociation from the putative RBP receptor, which is in accordance with previous observations by Sivaprasadarao and Findlay [25,26]. Using uterine brush-border membrane preparations, they found that bound RBP had a very high dissociation rate constant, with most of the bound RBP displaced from the receptor within 5 min. By increasing the pulse duration at 37 °C, or by lowering the temperature to 4 °C, much less RBP was released from the cells. These data suggest that the release process studied in the present report is only partly due to a rapid dissociation of RBP from its receptor. An additional

explanation for this fast release could be the recycling of internalized ligand from an early endosome.

Several kinetic studies show that many receptors are internalized within a few minutes at 37 °C and then recycled back to the cell surface with a half-life of 5 min [27]. Such internalization and recycling of RBP fits well with a recent study by Senoo et al. [7]. By using cryo-immunohistochemistry and electron microscopy they found that RBP was efficiently taken up by both liver parenchymal and stellate cells *in vivo*. At 10 min after injection, RBP was localized on the cell surface and in vesicles near the cell surface. RBP was mainly observed in association with the membrane in these vesicles. At 2 h after injection, RBP was also in larger vesicles located deeper in the cytoplasm of these cells. Together with the present study, these data suggest that RBP is internalized in parenchymal cells and that the internalization process is by receptor-mediated endocytosis.

Several other workers have presented evidence for a RBP receptor. Heller and Bok [12] reported that ^{125}I -RBP was bound only to the choroidal surface of the retinal pigment epithelial cells, and that an excess of unlabelled RBP greatly decreased the binding. McGuire and co-workers [28] observed that RBP bound primarily to interstitial cells of rat testes. Sivaprasadarao and Findlay [25,26], using placental brush-border membranes, found that the binding of ^{125}I -RBP had both high (3×10^{-9} M)- and low (9×10^{-8} M)-affinity binding components. These values agree well with those found in this report.

In two other studies using keratinocytes [29] and epithelial cells from the intestinal mucosa [10], no binding of ^{125}I -RBP to cells could be demonstrated. In these reports, repeated centrifugations to decrease unspecific cell-association was used, and hence specific RBP binding may have escaped their attention, due to the transient nature of the binding.

More recently, Båvik et al. [30] have attempted to characterize the RBP receptor in bovine retina pigment epithelial cells in more detail. Radiolabelled RBP was cross-linked with membranes, and complexes were analysed by electrophoresis and autoradiography. From such studies, the receptor was assumed to have a molecular mass of 63 000 Da [30].

In summary, the present study shows that different labelling techniques of RBP yield varying quantitative data when studying cellular binding, uptake and degradation. Therefore, in studies dealing with receptor-mediated endocytosis of RBP, care should be taken in choosing the methods for radiolabelling. From the results of this study, we would recommend the use of TC labelling in cellular- or intracellular-localization experiments. In most other experiments, the EB labelling would, in our opinion, be the most satisfactory and most consistent method. This

technique, coupled with the oil-centrifugation procedure, should provide an adequate experimental model for studying the binding of RBP to cells.

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