Solubilization and purification of the retinol-binding protein receptor from human placental membranes

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The membrane receptor for retinol-binding protein (RBP) has been solubilized from human placental brush-border membranes with octyl- β -glucoside, Nonidet P-40 and CHAPS. A method, based on the preferential precipitation of ^{125}I -RBP–receptor complex with poly(ethylene glycol) 8000, was developed in order to measure the RBP-binding activity in the detergent extracts. The receptor was fairly stable (4 °C, 7 days) in octyl- β -glucoside and Nonidet P-40, but quickly lost activity in CHAPS. The

detergent-solubilized form retained all the properties characteristic of the membrane-bound protein, except for a small decrease in affinity for RBP (3- and 7-fold in Nonidet P-40 and octyl- β -glucoside respectively). The receptor was isolated using recombinant RBP coupled to Reacti-Gel 6X affinity matrix. The purified material contained major and minor protein species of 63 and 55 kDa respectively on SDS/PAGE.

INTRODUCTION

Vitamin A is required for various physiological functions including vision, reproduction, growth and development. These functions are mediated by diverse classes of proteins, ranging from the membrane-bound G-protein-linked receptors (e.g. rhodopsin) to soluble DNA-binding transcription regulatory factors (retinoic acid receptors). Transport of vitamin A to target tissues from the liver stores is mediated by a specific plasma carrier protein, called retinol-binding protein (RBP). RBP is synthesized mainly in the liver, and secreted into the plasma as retinol–RBP complex. In the plasma, it circulates as a 1:1 complex with another protein called transthyretin (TTR) (for a recent review see Blomhoff et al., 1990).

Under normal nutritional conditions almost all circulating retinol is transported by RBP. The mechanism by which the vitamin is subsequently transported from RBP into vitamin A-requiring cells has been controversial. Some workers (Fex and Johanneson, 1988; Noy and Xu, 1990) believe that retinol, being extremely hydrophobic, does not utilize a specific transport mechanism but instead diffuses through the lipid bilayer of cell membranes of the target tissues. Others (Heller, 1975; Rask and Peterson, 1976; Sivaprasadarao and Findlay, 1988a,b; Bavik et al., 1991) have provided evidence for a specific RBP receptor, which under physiological conditions facilitates the uptake of retinol. The mechanism of this receptor-mediated delivery of retinol to the target tissues has not yet been defined. Experimental evidence provided by Heller (1975), Rask and Peterson (1976), Sivaprasadarao and Findlay (1988a,b) and Bavik et al. (1991) indicate that RBP binds to the cell-surface receptor, delivers retinol, loses its affinity for the receptor and then returns to the circulation. In contrast, Senoo et al. (1990), and more recently Matarese and Lodish (1993), demonstrated that retinol uptake involves internalization of RBP by receptor-mediated endocytosis. These differences may reflect different cellular functions or experimental conditions. Thus purification to homogeneity of the RBP receptor, with retention of biological activity, is crucial

to demonstrate unequivocally the validity of the previous observations. In this study we report the detergent solubilization and purification of the RBP receptor from human placental membranes using a recombinant RBP-affinity resin and demonstrate that the purified receptor exhibited all the properties that are characteristic of the membrane-bound receptor.

EXPERIMENTAL

Materials

Octyl- β -D-glucoside, Nonidet P-40, CHAPS, poly(ethylene glycol) 8000 (PEG 8000), dibutyl phthalate and goat γ -globulins were purchased from Sigma Chemical Co. Reacti-Gel 6X and Iodogen were obtained from Pierce Warriner. Enzymobeads was purchased from Bio-Rad Laboratories. Na ^{125}I was purchased from ICN Radiochemicals. All other reagents and chemicals were of appropriate grade and obtained from Sigma Chemical Co., or BDH Chemicals Ltd.

Preparation of RBP

RBP was purified from recently outdated human plasma as described previously (Sivaprasadarao and Findlay, 1988a). Recombinant RBP (rRBP) was purified from the periplasm of *Escherichia coli* overexpressing RBP (Sivaprasadarao and Findlay, 1993) using TTR–Sepharose CL 4B affinity resin as described by Vahlquist et al. (1971). The purified protein was stored at –20 °C in PBS (20 mM sodium phosphate, 150 mM NaCl, pH 7.4).

Preparation of placental membranes

Membrane vesicles were prepared from freshly delivered human placenta according to the method of Booth et al. (1980), and stored as 1 ml aliquots at –70 °C in PBS at a 5 mg/ml protein concentration.

Abbreviations used: RBP, retinol-binding protein; rRBP, recombinant RBP; PMSF, phenylmethanesulphonyl fluoride; PEG 8000, poly(ethylene glycol) 8000; CMC, critical micellar concentration; TTR, transthyretin.

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Assay of specific ^{125}I -RBP binding to placental membranes

Plasma or rRBP was labelled to a specific radioactivity of 150–250 Ci/mmol with ^{125}I using Enzymobeads according to the instructions of the manufacturer. Specific binding of ^{125}I -RBP to the membrane vesicles was performed using the oil centrifugation assay described previously (Sivaprasadarao and Findlay, 1988a).

Detergent solubilization of ^{125}I -RBP-binding activity

Placental membranes were suspended in 20 mM sodium phosphate/150 mM NaCl/2 mM MgCl_2 /0.2 mM phenylmethanesulphonyl fluoride (PMSF), pH 7.4, containing 1% (w/v) octyl glucoside or Nonidet P-40, to a final concentration of 5 mg of protein/ml. The mixture was drawn through a 22-gauge syringe needle repeatedly and kept for 30 min at room temperature with occasional stirring. After centrifugation at 105 000 *g* for 60 min at 4 °C to sediment the detergent-insoluble material, the supernatant was divided into aliquots and stored at –20 °C or used immediately for ^{125}I -RBP-binding-activity measurements.

Assay for soluble ^{125}I -RBP-binding activity

Solubilized membrane protein (20–100 μg) was mixed with ^{125}I -RBP [(5–10) $\times 10^4$ c.p.m.] in a total volume of 100 μl of PBS in 0.5-ml Eppendorf tubes. After incubation at room temperature for 15 min, 50 μl of ice-cold 5 mg/ml goat γ -globulins in 0.1 M sodium phosphate, pH 7.4, was added, followed by 100 μl of 25% (w/v) PEG 8000. The samples were immediately mixed thoroughly and incubated on ice for 25 min to ensure complete precipitation of the ^{125}I -RBP–receptor complex. The mixtures were centrifuged at 12 000 *g* for 8 min at 4 °C, overlaid to the top of the tubes with ice-cold dibutyl phthalate, and centrifuged for an additional 2 min. The aqueous supernatant and the oil layers were carefully aspirated off, and the tubes left inverted on a paper towel to drain any excess liquid. The bottoms of the tubes containing the pellets were cut off and counted for radioactivity in an LKB Rackgamma II counter. Non-specific binding was determined by the addition of excess (5 μM) unlabelled RBP to parallel reaction mixtures. Incubations (background) in the absence of membrane extract (but with the solubilization medium present) were also carried out to determine non-specific precipitation of ^{125}I -RBP by PEG 8000. All values were corrected by subtracting the background level. The assays were carried out in triplicate.

Assay of protein

The protein content of membranes was estimated by the method of Lowry et al. (1951) and that of detergent-soluble extracts as described by Markwell et al. (1978).

Radioiodination of placental membrane proteins

Labelling of membrane vesicles (10 mg) with ^{125}I (1 mCi) was carried out using Iodogen as described by Markwell and Fox (1978). The labelled membranes were thoroughly washed with PBS to remove unincorporated ^{125}I , and then solubilized in 1 ml of 2% octyl glucoside in PBS containing 2 mM MgCl_2 and 0.2 mM PMSF. Undissolved material was removed by centrifugation at 12 000 *g* for 15 min.

Affinity purification of RBP receptor

Plasma-derived human RBP or bacterially produced recombinant human RBP were attached to 1,1-carbonyldi-imidazole-

activated Sepharose CL 6B (Reacti-Gel 6X) according to the instructions of the manufacturer. Typically, 1 g of wet resin cake was transferred to a polypropylene tube containing 2.0 mg of RBP with a tracer amount of ^{125}I -RBP (1×10^6 c.p.m.) in 2.0 ml of 0.1 M borate, pH 8.5. The gel beads were then gently mixed on a Spiramix at 4 °C for 24 h. Monoethanolamine, adjusted to pH 9.0, was added to a final concentration of 1 M and the mixture incubated for 2 h at room temperature. The beads were then washed extensively using, successively, 100 ml each of the following: 50 mM Tris/1 M NaCl, pH 7.4; 0.1 M sodium phosphate/6 M urea, pH 7.4; 20 mM sodium phosphate/0.15 M NaCl/0.2 mM PMSF, pH 7.4; and finally with 0.5% octyl glucoside in 20 mM sodium phosphate/0.15 M NaCl/0.2 mM PMSF, pH 7.4 (buffer A). The washed gel was counted for bound radioactivity and the percentage of RBP coupled to the resin calculated. The uncoupled RBP present in the filtrate was also estimated by measuring absorbance at 280 nm after extensive dialysis. The coupling efficiency ranged from 60 to 80%. Half of the coupled resin was packed into a Pasteur pipette. The rest was mixed with the octyl glucoside extract of placental membranes made from two placentae (approx. 120 mg of protein), together with approx. 1×10^7 c.p.m. of the soluble extract of ^{125}I -labelled membrane proteins, and incubated at room temperature for 15 min with gentle mixing. The gel suspension was cooled to 4 °C and the resin allowed to settle. All operations henceforth were performed at 4 °C. The supernatant was removed, and the settled gel was packed on the top of the resin in the column. The packed resin was washed with buffer A until the radioactivity of the eluate reached background levels. The resin was then washed successively with five column volumes each of buffer B (20 mM sodium phosphate/1 M NaCl/0.5% octyl glucoside/0.2 mM PMSF, pH 7.4) and buffer A. Elution of the RBP receptor was achieved by successive washing with 0.5% octyl glucoside and 0.2 mM PMSF in distilled deionized water (buffer C) and 50 mM sodium acetate/1 M NaCl/0.5% octyl glucoside/0.2 mM PMSF, pH 5.0 (buffer D).

Fractions (500 μl) eluted with buffer D were collected into tubes containing 250 μl of 0.5% octyl glucoside in 0.3 M sodium phosphate, pH 7.4, in order to neutralize the eluate. All fractions were equilibrated with buffer A by dialysis and assayed for radioactivity (a measure of protein) and for ^{125}I -RBP-binding activity.

RESULTS

Solubilization of ^{125}I -RBP-binding activity

In the presence of octyl glucoside, 10% PEG 8000 did not precipitate significant amounts of free ^{125}I -RBP. However, after

Table 1 Effect of wash solution on the precision of ^{125}I -RBP binding assay

The octyl glucoside extract of membranes (20 μg of protein) was mixed with 6×10^4 c.p.m. of ^{125}I -RBP. After incubation at 22 °C for 15 min, 50 μl of ice-cold 5 mg/ml γ -globulins and 100 μl of 25% PEG 8000 solution were added. Following incubation on ice for 25 min, the suspension was centrifuged and the pellets were washed with dibutyl phthalate as described in the Experimental section (a) or superficially with 8% PEG 8000 solution (b). Values represent means $\pm$ S.D. ($n = 3$).

Wash solution	Radioactivity (c.p.m. bound)			
	Total	Non-specific	Background	Specific
(a) Dibutyl phthalate	4082 $\pm$ 45	2898 $\pm$ 44	2674 $\pm$ 45	1184 $\pm$ 63
(b) 8% PEG solution	3989 $\pm$ 488	2821 $\pm$ 244	2802 $\pm$ 4	1168 $\pm$ 545

Table 2 Solubilization of RBP receptor with various detergents and the stability of the receptor

Detergents were added to the membranes to the final levels indicated, keeping the concentration of membranes at 5 mg of protein/ml. The extracts, prepared as described in the Experimental section, were assayed for specific binding activity immediately, and after storage for 7 days at 4 °C.

Detergent	Concn.	Protein solubilized (%)	Activity solubilized (%)	Activity after 7 days at 4 °C (%)
Octyl glucoside	40 mM	38.6	106	84
Nonidet P-40	1%	50.8	90.9	100
CHAPS	1%	52.6	88.6	0

Figure 1 Precipitation of ^{125}I -RBP-receptor complex with PEG 8000

Octyl glucoside- (16 μg ; circles) or Nonidet P-40- (18 μg ; squares) solubilized placental membrane protein was incubated with 3.2×10^4 c.p.m. of ^{125}I -RBP in the presence (non-specific binding) or absence (total binding) of 5 μM unlabelled RBP. After incubation at room temperature for 15 min, 50 μl of 5 mg/ml γ -globulins in 0.1 M sodium phosphate buffer, pH 7.4, and 100 μl of PEG 8000 solution were added to the final concentrations indicated. Samples were then processed as described in the Experimental section. Specific binding ($\circ$, $\square$) was determined by subtracting non-specific ($\bullet$, $\blacksquare$) from the total binding. Values represent means $\pm$ S.D. ($n = 3$).

incubation with the octyl glucoside extract of placental membranes PEG 8000 precipitated much more ^{125}I -RBP, and this was inhibited by the inclusion of excess unlabelled RBP in the incubation mixture. Thus PEG 8000 can be used to precipitate the ^{125}I -RBP specifically bound to its receptor at a concentration lower than that required to precipitate unbound/free ^{125}I -RBP. As the RBP-receptor complex has a short half-life and dissociates rapidly if washed with aqueous buffers (Sivaprasadarao and Findlay, 1988a; Bavik et al., 1991), the PEG 8000 precipitate was recovered by centrifugation, the mixture overlaid with dibutyl phthalate and centrifuged again. This allowed the aqueous layer containing the unprecipitated ligand to rise through the denser oil layer, thus achieving physical separation of almost all the free

Figure 2 Effect of detergent concentration on the solubilization of ^{125}I -RBP-binding activity and total protein

Aliquots of placental membranes (5 mg of protein/ml) were incubated for 15 min at room temperature with detergent at the concentrations shown. After centrifugation at 105000 g for 1 h at 4 °C, the supernatant was assayed for its protein content and for ^{125}I -RBP-binding activity. Recoveries of the specific binding activities with Nonidet P-40 ($\circ$) and octyl glucoside ($\bullet$) and of total protein [Nonidet P-40 ($\square$), octyl glucoside ($\blacksquare$)] are expressed as a percentage of original membrane binding activity or protein. Values represent means of triplicate analyses.

ligand from precipitated ^{125}I -RBP without perturbing the equilibrium (Table 1). Using this assay, we were able to optimize conditions further for solubilization and assay of the receptor. We found that the presence of the Mg^{2+} ions is necessary for maximal recovery of the active receptor. Nonidet P-40 and CHAPS were as effective as octyl glucoside for receptor solubilization, but in CHAPS the receptor was unstable and lost its binding activity completely upon storage at 4 °C for 7 days (Table 2). No significant loss of binding activity was noticed in octyl glucoside and Nonidet P-40 extracts under the same conditions.

Figure 1 shows that the optimal PEG 8000 concentration for precipitation of the ^{125}I -RBP-receptor complex with both octyl glucoside and Nonidet P-40 extracts was 10%. With Nonidet P-40 extracts, the non-specific binding was higher and consequently the S.D. values were slightly greater than with octyl glucoside. Background precipitation of ^{125}I -RBP, determined in the absence of the detergent extract, was about 5%.

Figure 2 shows the recovery of ^{125}I -RBP-binding activity and total protein as a function of detergent concentration for octyl glucoside and Nonidet P-40. Almost all (90–100%) of the total membrane-associated RBP-binding activity was solubilized when the concentrations of the detergent and the membrane protein were 1% and 0.5% respectively, suggesting that a detergent to protein ratio of 2:1 (w/w) is optimal for the solubilization of RBP-binding activity. For optimal activity, the concentration of octyl glucoside in the assay medium had to be maintained at or above 0.9% (Figure 3).

Figure 4 shows that the binding activity of both octyl glucoside and Nonidet P-40 extracts of the membranes increased with increasing protein content, indicating that the binding activity is membrane-associated.

Figure 3 Specific binding of ^{125}I -RBP to solubilized RBP receptor as a function of octyl glucoside concentration in the assay

Each assay contained $20\text{ }\mu\text{g}$ of solubilized membrane protein and 5.2×10^4 c.p.m. of ^{125}I -RBP, together with the concentration of octyl glucoside indicated. Values represent means of triplicate analyses.

Figure 4 Dependence of specific ^{125}I -RBP-binding activity on the amount of solubilized protein

Various amounts of octyl glucoside- (○) or Nonidet P-40- (●) solubilized membrane protein were incubated with 5.1×10^4 c.p.m. of ^{125}I -RBP and their binding activities determined as described in the Experimental section. Values represent means of triplicate analyses.

Analysis of equilibrium binding

Solubilization of several receptors including those for insulin (Gould et al., 1981), opiates (Simonds et al., 1980) and angiotensin II (Capponi et al., 1983), revealed that although recovery of receptors in their active form can be achieved with various detergents, their kinetic parameters are preserved in only some. Moreover, the binding parameters appear to be influenced by the detergent concentration in the assay medium (Gould et al., 1981). We therefore examined the binding parameters of the octyl glucoside- and Nonidet P-40-solubilized forms of the RBP

Figure 5 Scatchard analysis of ^{125}I -RBP binding to detergent extracts of placental membranes

Competitive inhibition analyses of ^{125}I -RBP binding by various concentrations of unlabelled RBP to (a) octyl glucoside and (b) Nonidet P-40 extracts were performed and the data transformed to Scatchard form. The data (means of triplicate analyses) were fitted by linear regression analysis at the 95% confidence limit. The calculated K_D and B_{max} values for binding at 15 mM octyl glucoside concentration (●, below CMC) are $(5.9 \pm 0.93) \times 10^{-7}$ M and 1.32 ± 0.13 pmol/mg of soluble protein respectively; and at 38 mM (○, above CMC) $(6.0 \pm 0.4) \times 10^{-7}$ M and 1.96 ± 0.08 pmol/mg of soluble protein respectively (a). For the Nonidet P-40 extract (b), the K_D and B_{max} values are $(2.7 \pm 0.24) \times 10^{-7}$ M and 0.63 ± 0.04 pmol/mg of soluble protein respectively.

receptor, with different concentrations of the detergent in the assay medium. From Scatchard analysis, the K_D values for the binding processes (Figure 5a) occurring below (15 mM) and above (38 mM) the critical micellar concentration (CMC) of octyl glucoside were $(5.9 \pm 0.93) \times 10^{-7}$ M and $(6.0 \pm 0.4) \times 10^{-7}$ M respectively. The B_{max} obtained with octyl glucoside concentrations above the CMC (1.96 ± 0.08 pmol/mg of soluble protein) was 1.5-fold higher than that obtained at sub-CMC concentrations (1.32 ± 0.13 pmol/mg of soluble protein). With the Nonidet P-40-solubilized extract (Figure 5b), the K_D and B_{max} values are $(2.7 \pm 0.24) \times 10^{-7}$ M and 0.63 ± 0.04 pmol/mg of the soluble protein respectively.

Figure 6 Specificity of binding

^{125}I -RBP-binding activity of the octyl glucoside extract of membranes was performed in the presence of the indicated concentrations of RBP ($\circ$), human serum albumin ($\bullet$), TTR ($\square$) or β -lactoglobulin ($\blacksquare$). The results are expressed as the percentage of binding activity relative to binding activity in the absence of added competing protein (100%). Values represent means of triplicate analyses.

Table 3 Effect of NaCl concentration on the ^{125}I -RBP-binding activity of placental membranes

Placental membranes were washed with the assay buffers of appropriate pH (20 mM sodium phosphate, pH 7.4 and 8.0, and 50 mM sodium acetate, pH 5.0), and ionic strength and assayed in the same buffers for ^{125}I -RBP-binding activity as described previously (Sivaprasadarao and Findlay, 1988a).

NaCl concn. pH...	Specific binding (%)		
	5.0	7.4	8.0
None	98	5.4	7.6
0.15	12	100	87
1.0	8.0	85	79

Specificity

The specificity of the solubilized RBP receptor was examined by testing the abilities of RBP and other proteins to inhibit ^{125}I -RBP-binding activity (Figure 6). As observed previously with the membrane-bound receptor (Sivaprasadarao and Findlay, 1988a), neither serum albumin nor β -lactoglobulin inhibited ^{125}I -RBP-receptor interaction. TTR showed an apparent increase in non-specific binding, which can probably be attributed to the ability of PEG 8000 to precipitate more ^{125}I -RBP when bound to TTR than when free. Under such conditions, any inhibition expected from the presence of TTR would be obscured.

Affinity chromatography of RBP receptor

Our initial purification procedures made use of serum RBP as the affinity ligand. RBP was coupled to Reacti-Gel 6X affinity resin, which was incubated with the octyl glucoside extract of placental membrane vesicles before packing into a small column. A sample (1×10^7 c.p.m.) of an octyl glucoside extract of ^{125}I -labelled

Figure 7 Affinity chromatography of RBP receptor on RBP-affinity resin

An octyl glucoside extract of human placental membranes (120 mg of protein), mixed with 1×10^7 c.p.m. of ^{125}I -labelled membrane extract was purified on RBP-Reacti-Gel 6X affinity matrix as described in the Experimental section. Arrows indicate positions where various buffers (A–D) were introduced to the column. The compositions of various buffers used are: A, 20 mM sodium phosphate/150 mM NaCl/0.5% octyl glucoside/0.2 mM PMSF, pH 7.4; B, 20 mM sodium phosphate/1 M NaCl/0.5% octyl glucoside/0.2 mM PMSF, pH 7.4; C, 0.5% octyl glucoside/0.2 mM PMSF in deionized water; and D, 50 mM sodium acetate/1 M NaCl/0.5% octyl glucoside/0.2 mM PMSF, pH 5.0.

placental membranes was added to the solution to allow the elution profile of membrane proteins to be monitored. After washing with equilibration buffer (buffer A, see the Experimental section) until the radioactivity reached background level, the column was washed with five column volumes of high-salt buffer (buffer B), which appears to stabilize the binding of the receptor to the resin while removing some non-specific contaminant proteins held to the resin by ionic interactions.

The conditions for elution of the receptor were determined from studies with the membrane-bound receptor. It was found that in the absence of salt at neutral pH RBP binding to the receptor was negligible, and in the presence of 1.0 M NaCl, at pH 5.0, virtually no ^{125}I -RBP bound to the receptor (Table 3).

Attempts to elute the bound receptor were therefore performed first with deionized water (buffer C) and then with 50 mM acetate buffer, pH 5.0, containing 1 M NaCl (buffer D) both of which contained 0.5% octyl glucoside. The yield of the receptor protein in this protocol was low, as judged from ^{125}I -RBP-binding activity of the eluted fractions as well as SDS/PAGE. The poor yield was not surprising for two reasons: first, RBP dissociates from the receptor rapidly upon dilution ($t_{1/2} = 4$ min) and secondly, serum RBP contains a large proportion of RBP that is not recognized by the receptor (Sivaprasadarao and Findlay, 1993).

To improve the yield, two modifications were made. First, the resin equilibrated with the crude extract of the receptor was packed on the top of a cushion of fresh RBP-affinity resin in order to ensure that any receptor that dissociates from the top resin during washing was retained by the lower cushion. Secondly, we used rRBP, expressed from *E. coli*, in view of its superior receptor-binding activity (Sivaprasadarao and Findlay, 1993). These modifications markedly improved the yield of the receptor, as judged from the binding activities of the eluted fractions (Figure 7). The incomplete release of the receptor with buffer C was made good with buffer D. The pool of the eluted fractions

Figure 8 SDS/PAGE of purified RBP receptor

One tenth of the pooled fractions (fractions 16 to 24 from Figure 7) were loaded. Positions of the molecular-mass-marker proteins are indicated on the left.

showed a major 63 kDa and a minor 55 kDa component on SDS/PAGE (Figure 8).

DISCUSSION

The results demonstrate that the RBP receptor has been solubilized by octyl glucoside treatment of the membranes prepared from human placental brush border. The binding activity could not be sedimented from this detergent at 105000 *g*. The solubilized receptor retained its activity to form a specific complex with ^{125}I -RBP. The ability of 10% PEG 8000 to precipitate this complex in preference to free ^{125}I -RBP allowed us to develop an assay for the soluble receptor. The previous PEG-based procedures for soluble receptor assays (Cuatrecasas, 1972) involved washing the precipitated labelled ligand-receptor complex with an aqueous PEG solution to reduce the background. As the ^{125}I -RBP-receptor complex is known to be unstable under these conditions due to its fast dissociation kinetics (Sivaprasadarao and Findlay, 1988a), we substituted a non-aqueous dibutyl phthalate wash for aqueous PEG. This minor modification allowed accurate and reproducible assay of the soluble form of the RBP receptor and can be extended to the analysis of other fast-dissociating ligand-receptor interactions.

The receptor was fairly stable in octyl glucoside and Nonidet P-40 solution, but quickly lost its binding activity when extracted and stored in CHAPS. The receptor thus appears to require a non-ionic detergent for stability. The necessity for detergent suggests that the protein is deeply embedded in the lipid bilayer. For optimal solubilization, Mg^{2+} ions are required. The requirement of Mg^{2+} ions for optimal solubilization has also been reported for opiate receptors (Demoliou-Mason and Barnard, 1984). For optimal binding activity, the RBP receptor required a detergent concentration that is greater than the CMC, an observation that has been reported for prolactin (Liscia et al.,

1982) and opiate (Simonds et al., 1980) receptors and interpreted as indicating a lipid environment for functional stability. Scatchard analysis (Figure 5) of ^{125}I -RBP binding to the receptor revealed that increasing the detergent concentration further did not affect the affinity of the receptor for RBP. Instead, an increase in the number of available binding sites occurred. This suggested that at low detergent concentrations, aggregation of the receptor might occur, as suggested for epidermal growth factor receptor (Carpenter, 1985). Addition of exogenous phospholipid was not required for preservation of biological activity (results not shown). Scatchard analysis (Figure 5) revealed that, compared with the membrane-bound receptor ($K_D = 0.79 \times 10^{-7}$ M, Sivaprasadarao and Findlay, 1988a), the solubilized receptor showed a 7-fold decrease in the affinity for RBP in octyl glucoside ($K_D = 6.0 \times 10^{-7}$ M) and a 3-fold decrease in Nonidet P-40 ($K_D = 2.7 \times 10^{-7}$ M). In other respects, including specificity (Figure 6) and sensitivity to *p*-chloromercuribenzenesulphonate (results not shown), the soluble form of the receptor is indistinguishable from the membrane-bound form. β -Lactoglobulin, a close structural analogue of RBP, failed to inhibit ^{125}I -RBP binding to the soluble receptor, suggesting that its strict specificity is preserved upon solubilization. Using a RBP-Reacti-Gel 6X affinity resin, we purified the RBP receptor from an octyl glucoside extract of placental membranes. The ^{125}I -RBP binding affinity was eluted with 0.5% octyl glucoside in deionized water. The inability of the receptor to bind RBP at low ionic strength suggested that binding may involve hydrophobic interactions. This is consistent with our site-directed-mutagenesis study which showed that it is by the hydrophobic residues of its loops that RBP mainly binds to the receptor (Sivaprasadarao et al., 1993). The yield of the receptor binding activity with serum-derived RBP-affinity resin, however, was low. This was not unexpected in view of the poor stability of serum RBP and its receptor complex. By using rRBP for the affinity resin and employing a cushion of fresh rRBP-affinity resin in the column, we have been able to improve the yield about 10-fold. We have previously shown that rRBP, expressed in *E. coli*, binds to the receptor about 6- to 8-fold better (higher B_{max}) than the plasma-derived RBP (Sivaprasadarao and Findlay, 1993). The purified preparation showed two components on SDS/PAGE, a major 63 kDa band and a minor 55 kDa band. Although, we are not certain at this stage whether one or both of these bands constitute the receptor protein, the recent demonstration by Bavik et al. (1991) that RBP can be chemically cross-linked specifically to a 63 kDa protein in bovine pigment epithelial cell membranes suggests that the 63 kDa band of our preparation is more likely to be the RBP-binding species. The apparently non-stoichiometric amount of 55 kDa component (as judged from the intensity of the stained bands on the gel) also tends to suggest that it could be a contaminant or a proteolysed form of the 63 kDa species, but it is not possible at this stage to eliminate the possibility that it could be a subunit of the receptor protein. Bavik et al. (1992) reported the isolation of a 63 kDa protein by immunoaffinity chromatography using an anti-p63 monoclonal antibody (A52) as the ligand. More recently Bavik and co-workers cloned the cDNA for this protein using the monoclonal antibody as a probe (Bavik et al., 1993). Northern-blot analysis failed to detect the transcript of the 63 kDa protein in tissues other than the pigment epithelium. This is surprising in view of the reports that RBP receptor is widely distributed in a number of tissues, notably the testis (for a review see Blomhoff et al., 1990). Moreover, the amino acid sequence deduced from the cDNA sequence did not indicate an N-terminal signal sequence or obvious transmembrane domains. The cRNA transcript of the clone, when expressed *in vivo* in a rabbit reticulocyte system

appeared in the lysate rather than associating with the microsomal fraction. The RBP receptor purified by us, on the contrary, is an integral membrane protein, which is consistent with the fact that all the currently known transporters, channels and receptors mediating endocytosis of nutrients are integral membrane proteins. Thus until the cDNA clone isolated by Bavik et al. (1993) is expressed in mammalian cells and the RBP-binding activity demonstrated, the identity of the clone remains uncertain.

The availability of the purified receptor, the function of which can be examined by the soluble receptor assay in detergent solution, offers an opportunity for studying the molecular mechanism of the receptor-mediated transfer of retinol from RBP to the interior of the cell. In addition, amino acid sequence analysis on the purified receptor protein should allow the design of oligonucleotide probes for cloning. This in turn will allow structure-function studies on the receptor using protein engineering.

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