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The interaction of retinol-binding protein with its plasma-membrane receptor

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^{125}I-labelled retinol-binding protein (RBP) bound to specific receptors in human placental brush-border membranes. Binding at 22 °C reached equilibrium within 15 min, but prolonged incubation caused a subsequent decline. Scatchard analysis of the equilibrium binding data at 22 °C and 15 min showed high-($3.0 \pm 2.7 \times 10^{-9}$ M) and low-($9.5 \pm 3.5 \times 10^{-8}$ M) affinity binding components. ^{125}I-RBP, bound to membranes at 22 °C for 15 min and subsequently dissociated with excess unlabelled RBP, exhibited biphasic dissociation kinetics consisting of fast and slow components of release. In contrast, Scatchard analysis and dissociation kinetics of the binding that had taken place at 37 °C for 1 h showed the fast-dissociating/low-affinity binding component, but little of the slow-dissociating/higher-affinity binding component. When ^{125}I-RBP, after incubation with membranes at 37 °C for 1 h, was re-isolated and subjected to dissociation kinetic analysis using a fresh batch of membranes, the fast-dissociating phase was unchanged, but the slow phase was almost absent. The complex kinetics were interpreted in terms of a heterogeneity in RBP consisting of high- and low-affinity binding forms. The higher-affinity-binding form is thought to be converted into the lower-affinity state on binding to the receptor. Transthyretin inhibited ^{125}I-RBP binding to the membrane, suggesting that free, rather than transthyretin-associated, RBP bound to the receptor. The RBP receptor was trypsin-, heat- and thiol-group-specific-reagent sensitive and was highly specific for RBP.

INTRODUCTION

Vitamin A is transported in plasma as retinol bound to retinol-binding protein (RBP). Intracellularly, retinol is also found as a complex, but with a different protein, called 'cellular retinol-binding protein'. Several roles have been suggested for the evolution of these specific extra- and intra-cellular transport mechanisms (for a review, see Wolf, 1984). As the interface between plasma and the cytoplasm, plasma membranes are a critical part of the transport system for retinol, but the mechanism(s) *in vivo* by which the vitamin is transferred from extracellular RBP to the intracellular binding proteins has not yet been rigorously established. Specific uptake of RBP-bound retinol has been demonstrated in cells from bovine pigment epithelium (Chen & Heller, 1977), monkey small intestine (Rask & Peterson, 1976), cornea (Rask *et al.*, 1980) and liver parenchyma (Blomhoff *et al.*, 1985). From these studies it was suggested that cellular uptake of retinol involves the interaction of RBP with a plasma-membrane receptor of some kind. However, direct binding of RBP could be demonstrated only in pigment epithelial cells (Heller, 1975), and even here the specificity and characteristics of the binding were not clearly defined.

As part of an investigation to describe in detail the steps involved in the delivery of retinol from RBP to target cells, studies on the interaction of ^{125}I-labelled RBP with plasma membranes have been carried out. Membranes isolated from the human placental syncytiotrophoblast were used principally because this represented the only known route for the delivery of vitamin A to the foetus (Takahashi *et al.*, 1977).

MATERIALS AND METHODS

Materials

Carrier-free Na^{125}I was obtained from Amersham International. Crystalline human serum albumin, egg albumin, transferrin and trypsin (935 units/mg) were purchased from Sigma Chemical Co. Soybean trypsin inhibitor (7756 units/mg) was from Calbiochem. PCMBS (sodium salt), iodoacetate (sodium salt), iodoacetamide, NEM DTT and PMSF were also from Sigma. Dibutyl phthalate was from Aldrich Chemical Co., and dinonyl phthalate was from BDH. Enzymobeads were purchased from Bio-Rad Laboratories. DDISA was synthesized as described by Barclay & Findlay (1984). Urinary RBP (isolated from the urine of proteinuric patients) was a gift from Professor E. Cooper, Unit for Cancer Research, University of Leeds, Leeds, U.K. All other chemicals were of reagent grade. Human placentae were obtained from Leeds General Infirmary, Leeds, U.K. Human RBP and transthyretin (TTR) were purified to homogeneity from plasma by using the methods described previously (Rask *et al.*, 1971; McGuire & Chytil, 1980).

Preparation of placental microvilli

Microvilli were isolated from recently delivered normal human term placentae according to the method of Booth *et al.* (1980). The alkaline phosphatase activity

Abbreviations used: DDISA, 3,5-di-iodo-4-diazobenzenesulphonic acid; DTT, dithiothreitol; HSA, human serum albumin; NEM, *N*-ethylmaleimide; PCMBS, *p*-chloromercuribenzenesulphonic acid; PMSF, *p*-phenylmethanesulphonyl fluoride; RBP, retinol-binding protein; TTR, transthyretin.

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of the final preparation was increased 20–25-fold over that of the initial homogenate. Membranes were stored in portions at −20 °C. Before use, they were thawed at room temperature, incubated at 37 °C for 15 min, and washed twice with the binding assay buffer at 4 °C.

Radioiodination of RBP

RBP was labelled with ^{125}I by using Enzymobeads according to the instructions of the manufacturer. Free ^{125}I was separated from radioiodinated RBP by using a Sephadex G-75 column (60 cm × 0.9 cm). The specific activity of the labelled RBP ranged from 150 to 230 Ci/mmol, with more than 97% of the radioactivity being precipitable at 4 °C by 10% trichloroacetic acid.

Binding of ^{125}I-RBP to microvilli

Binding assays were performed by a phthalate/oil-separation method (Cuatrecasas & Hollenberg, 1976; Sivaprasadarao & Findlay, 1987). Membranes (1–2 mg of protein/ml) were incubated with ^{125}I-RBP (2–10 nM) in 150 μl of 0.1% ovalbumin, 20 mM-sodium phosphate and 150 mM-NaCl, pH 7.4 (binding assay buffer). After incubation at the desired temperature for the desired length of time, a 100 μl aliquot of the incubation mixture was transferred to precooled 300 μl-capacity polypropylene centrifuge tubes (Sarstedt 72/702) containing dibutyl phthalate/dinonyl phthalate (3:2, v/v). Membrane-bound ^{125}I-RBP was separated from unbound ^{125}I-RBP by centrifugation for 2 min at 12500 ***g***. The bottom of the tube containing the pellet was cut off and counted for radioactivity in an LKB 1270 Rackgamma II liquid-scintillation counter. Non-specific binding of ^{125}I-RBP was measured in the presence of at least 2 μM unlabelled RBP. Binding and kinetic experiments were carried out between 5 and 20 times.

Association kinetics

The rapid association of RBP with the membranes necessitated that each time point be determined individually. Incubations (assay volume 100 μl) were carried out on top of the phthalate/oil mixture, since it was found that the phthalate esters did not interfere with the binding assays. Incubation medium (70 μl) containing all the components except membranes were layered on the top of 200 μl of the phthalate/oil mixture and spun for about 30 s to obtain a clean oil/aqueous interphase. The binding reaction was started by the addition of 30 μl of membranes (preincubated for 2 min at the assay temperature) to the preincubated reaction mixture with sufficient speed to ensure mixing. Incubations were terminated by centrifugation. Non-specific binding for each association time point was determined in a parallel set of incubations, in the presence of 2 μM-RBP.

Dissociation kinetics

Membranes were equilibrated with ^{125}I-RBP by incubating for 15 min at 22 °C (total volume 2.0 ml). A 50 μl portion of this mixture was carefully layered on to the phthalate/oil mixture. Dissociation was monitored after the addition of 50 μl of unlabelled RBP (final concn. 2 μM). After incubation for the desired time at 22 °C, the dissociation reaction was terminated by centrifugation as described above. A 50 μl portion of buffer, instead of RBP, was added to one set of tubes to determine equilibrium binding at zero dissociation time. The binding remaining after 30 min was taken as non-specific binding, and this value was subtracted from all time points to obtain the specific ^{125}I-RBP binding remaining.

Protein determination

The protein content of the membranes was determined as described by Markwell *et al.* (1978), with bovine serum albumin as the standard.

Trypsin digestion

Membranes (4 mg/ml) were incubated with trypsin (0.2 mg/ml) for 60 min at 37 °C. The digestion was terminated by addition of soyabean trypsin inhibitor to a final concentration of 0.5 mg/ml.

Data analysis

The experimental data were analysed using a Fortran 77 computer program (Numerical Algorithms Group Subroutine E04GDF was used) to give a non-linear least-squares fit. The results were treated as follows.

Assuming a reversible reaction between the two forms of RBP (L), designated L_f (fast-associating RBP) and L_s (slow-associating RBP) and a single class of non-interacting receptors (R), under pseudo-first-order conditions (when $[L] \gg [R]$), the formation of L_fR and L_sR with time (t) approaching equilibrium (eq.) can be described by the equations:

$$(L_fR)_t = (L_fR)_{eq.}(1-e^{-k_f t}) \quad (1)$$

$$(L_sR)_t = (L_sR)_{eq.}(1-e^{-k_s t}) \quad (2)$$

where k_f and k_s are pseudo-first-order rate constants for the net formation of L_fR and L_sR respectively.

Total RBP (both forms) binding to the receptor at $t = (L_fR)_{eq.}(1-e^{-k_f t}) + (L_sR)_{eq.}(1-e^{-k_s t})$ (3)

If k_f is very large, so that the binding of fast-associating RBP to the receptor reaches equilibrium before the separation of free from the membrane bound RBP is achieved, then:

Total binding to the receptor at the experimental time point =

$$(L_fR)_{eq.} + (L_sR)_{eq.}(1-e^{-k_s t}) \text{ or } (LR)_{eq.} - (L_sR)_{eq.}e^{-k_s t}$$

where $(LR)_{eq.}$ is the concentration of total receptor-bound RBP at equilibrium and $(LR)_{eq.}$, $(L_sR)_{eq.}$ and $-k_s$ correspond to K_1, K_2 and K_3 of the computer equation (see under 'Association kinetics' in the Results section).

The dissociation of the RBP–receptor complex is expected to take a bi-exponential time course when RBP is present as fast-dissociating (L_f) and slow-dissociation (L_s) forms. The dissociation equations may then be written as:

$$(L_fR)_t = (L_fR)_{eq.}e^{-k_f t} \quad (6)$$

$$(L_sR)_t = (L_{fs}R)_{eq.}e^{-k_s t} \quad (7)$$

Thus: total RBP bound at time t of dissociation =

$$(L_fR)_{eq.}e^{-k_f t} + (L_sR)_{eq.}e^{-k_s r} \quad (8)$$

If k_f is very large, at zero dissociation time in the oil centrifugation assay, all L_fR will have dissociated and that remaining can be described by a single exponential, that is:

RBP bound at time t of dissociation $= (L_sR)_{eq.}e^{-k_s t}$ (9)

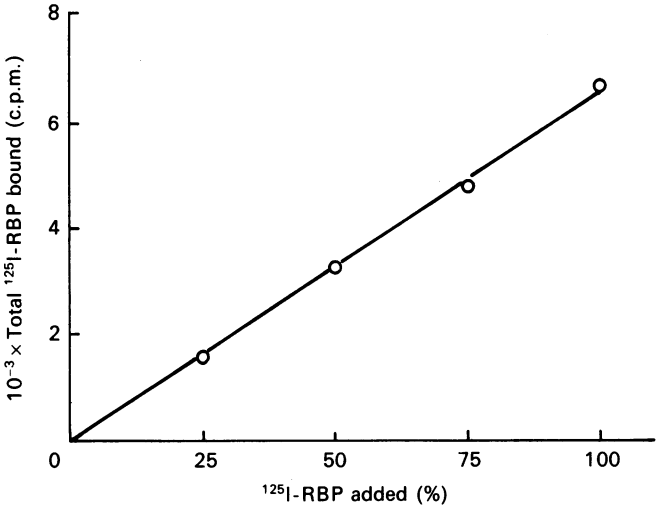

Fig. 1. Total binding of ^{125}I-RBP to the placental membrane vesicles

The proportion of unlabelled and labelled RBP was varied, whereas the concentration of total RBP (10 nM) and the amount of membranes (0.62 mg protein/ml) were kept constant. Binding was measured after 15 min at 22 °C.

The first-order rate plot does not pass through the origin, and the intercept on the ordinate is equal to the completely dissociated L_fR.

RESULTS

Characterization of ^{125}I-RBP

Analysis of ^{125}I-RBP by SDS/polyacrylamide-gel electrophoresis followed by autoradiography showed a single band with an apparent M_r of 21000. In order to show that the observed specific binding of ^{125}I-RBP to the placental microvilli is not due to a minor subpopulation of labelled material, and that the affinity of RBP to the microvilli was not altered by radioiodination, iodinated and unmodified RBPs were mixed in various proportions while maintaining the same total concentration of RBP (10 nM) before addition to the membranes at 22 °C. As shown in Fig. 1, a plot of ^{125}I-RBP bound to membranes against the percentage of ^{125}I-RBP added was linear, the line passing through the origin.

Time-, temperature- and pH-dependence of binding

Fig. 2 shows the temperature-dependence of ^{125}I-RBP binding to the placental membranes. At 0 °C, ^{125}I-RBP binding reached equilibrium very rapidly and was approx. 60 % of that at 22 °C. The binding reaction appeared to reach equilibrium within 15 min at 22 °C. In the presence of a 200-fold molar excess of unlabelled RBP, the total binding was diminished by about 80 %, indicating that unlabelled RBP and ^{125}I-RBP were competing for a limited number of binding sites. This non-specific binding was instantaneous, constant at all times and showed no temperature-dependence. The binding at 0 °C remained little altered for 3 h. In contrast, equilibrium binding at 22 °C decreased with time, reaching, after 3 h, a value approximately equal to that at 0 °C. Specific binding

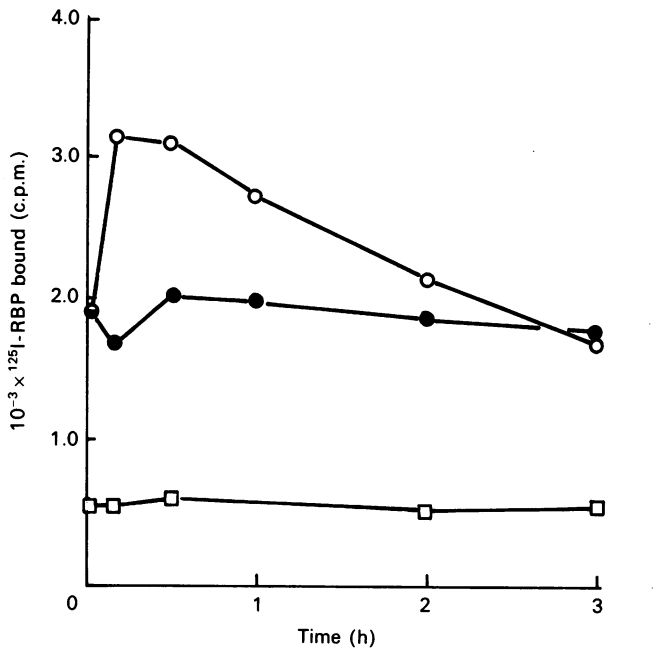

Fig. 2. Time- and temperature-dependence of ^{125}I-RBP binding to placental membrane vesicles

Membranes (0.64 mg of protein/ml) were incubated with ^{125}I-RBP (4.7 nM) at either 22 °C (○) or 0 °C (●). At the time points indicated, aliquots were removed and assayed for binding as described in the Materials and methods section. Non-specific binding at 22 °C (□) was determined in the presence of 2 μM unlabelled RBP.

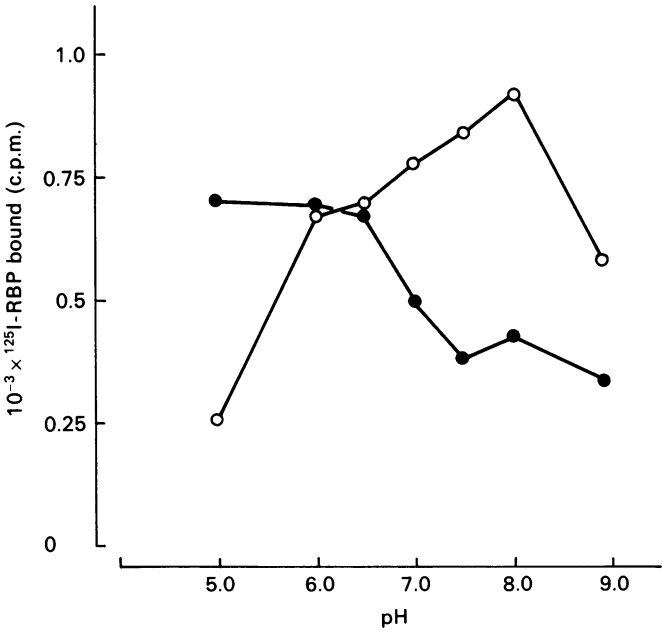

Fig. 3. pH optimum for binding of ^{125}I-RBP to placental membrane vesicles

Membranes (0.65 mg of protein/ml) were incubated with ^{125}I-RBP (2.5 nM) in the absence and presence of 2 μM-RBP and in the following buffers: sodium acetate, pH 5.0; sodium phosphate, pH 6.0–8.0 as shown; and Tris/HCl, pH 8.8. Final concentrations of the buffers and NaCl were 25 and 150 mM respectively. ○, Specific binding; ●, non-specific binding.

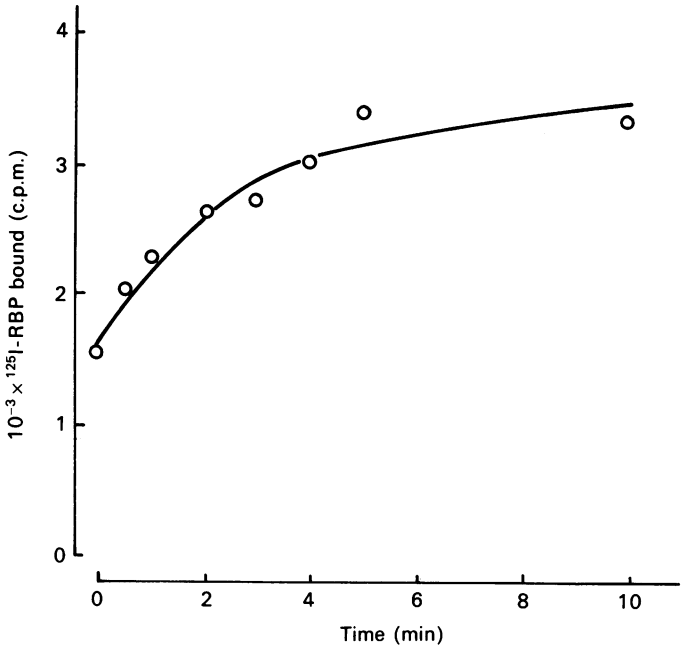

Fig. 4. Kinetics of association of ^{125}I-RBP to placental membrane vesicles

Specific ^{125}I-RBP binding at 22 °C, after the addition of membranes (0.76 mg of protein/ml) to ^{125}I-RBP (8 nM) is plotted against time. The continuous curve was calculated from the equation:

$$\text{Bound}_{t(\text{c.p.m.})} = K_1 + K_2 \cdot \exp(-K_3 t)$$

with the parameter values determined by least-squares fitting; $K_1 = 3370$ c.p.m., $K_2 = -1745$ c.p.m. and $K_3 = 0.439\ \text{min}^{-1}$. The coefficient of multiple correlation was 0.987.

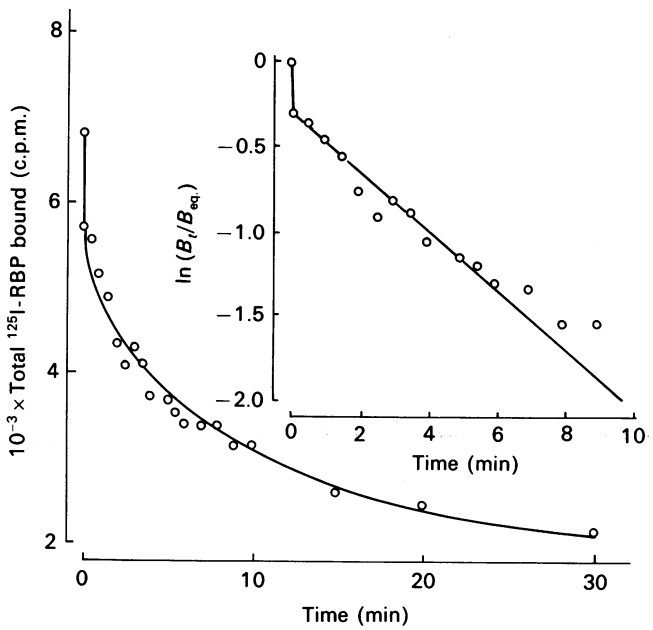

Fig. 5. Dissociation kinetics of ^{125}I-RBP binding

Membranes (1 mg of protein/ml) were incubated with ^{125}I-RBP (7.8 nM) at 22 °C to equilibrium. Dissociation was initiated by the addition of RBP (2 μM) and terminated at the times indicated by rapid centrifugation through oil. Bound ^{125}I-RBP remaining was plotted as a function of time. The inset shows the first-order disappearance of ^{125}I-RBP bound to membranes; data are from the main Figure between the experimental time points 0 and 10 min of dissociation and are plotted with $\ln(B_t/B_{eq.})$ on the ordinate and time on the abscissa, where B_t = binding remaining at time t, and $B_{eq.}$ = equilibrium binding. The slope of the curve is $-0.174 \pm 0.015\ \text{min}^{-1}$, the intercept -0.297 ± 0.08, and the standard error of the fit is 0.184.

decreased markedly below pH 7.0 and above pH 8.0 (see Fig. 3).

Association kinetics

Fig. 4 illustrates the kinetics of association of ^{125}I-RBP with placental membranes. The binding curve was biphasic, with very-fast- and slow-binding components. If the two phases of the curve were to represent two independent binding processes, the data should be described by an equation which is a sum of two exponential terms (see the Materials and methods section). However, when the data were analysed by non-linear least-squares analysis, the resulting curve fitted best the equation:

$$B_t = K_1 + K_2 \exp(-K_3 t)$$

where B_t is binding at time t, K_1 is total binding at equilibrium, K_2 is equilibrium binding due to the slow phase and K_3 is the pseudo-first-order rate constant. This could be explained by the fact that the fast-binding component, L_rF, which is equal to $K_1 + K_2$, had reached equilibrium before separation of membrane-bound ^{125}I-RBP from free ^{125}I-RBP could be achieved by the oil-centrifugation technique. As a result, the fast-binding component, which otherwise should have been described by an exponential-containing term, was reduced to a constant in the equation. The possibility that the overall binding process was due to single homogeneous slow-binding process, and that the fast-component resulted from binding that had occurred before the separation of bound and free ^{125}I-RBP, was eliminated by extrapolation of the initial part of the association curve. The resultant intercept at 3 min is more than the time used in the oil-centrifugation method. The pseudo-first-order rate constant for the slow association process was calculated as $0.439\ \text{min}^{-1}$.

Dissociation kinetics

The dissociation of ^{125}I-RBP from the membranes, induced by the addition of chase concentrations of unlabelled RBP also exhibited biphasic kinetics (Fig. 5). This again suggests that two binding components with different dissociation rates are involved. Approx. 25% of the bound ^{125}I-RBP was released almost instantaneously (fast phase) and the rest in a relatively slow phase. As a result, the data fitted a curve that showed a y-intercept equal to the rapidly dissociating component of ^{125}I-RBP binding, which had undergone complete dissociation before the separation of free from the membrane-bound ^{125}I-RBP was achieved. From the slope of the straight line, which represents the slow-dissociating binding component, a dissociation rate constant of $0.174\ \text{min}^{-1}$ was calculated.

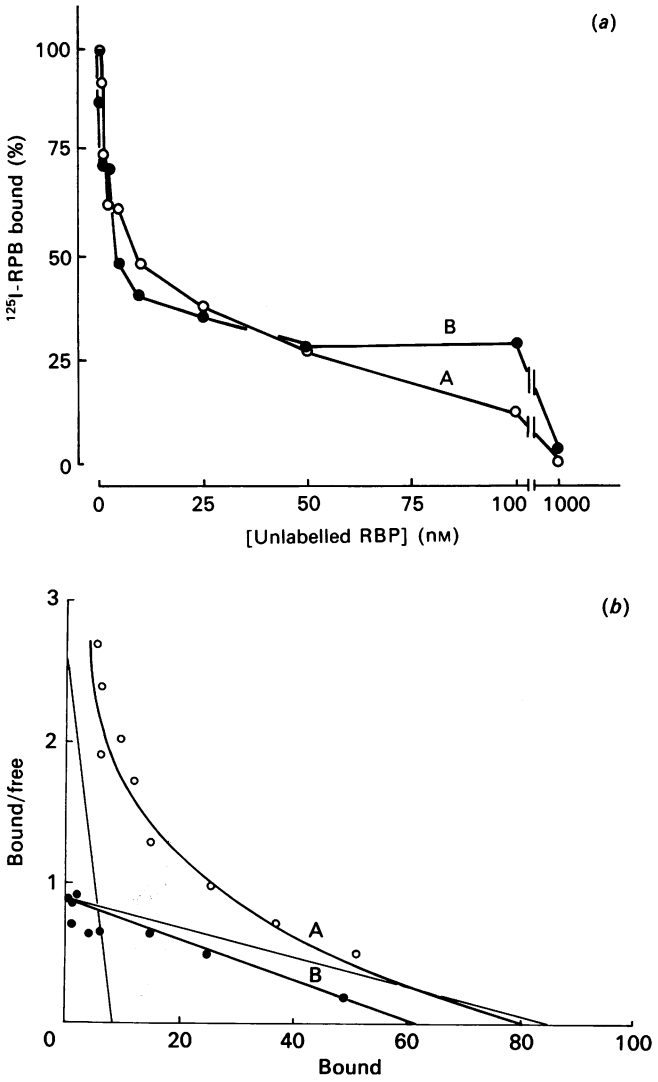

Fig. 6. (*a*) Competitive inhibition of ^{125}I-RBP binding to placental membrane vesicles by unlabelled RBP and (*b*) Scatchard analysis of the binding data

(*a*) Membranes (0.5 mg/ml) were incubated at 22 °C for 15 min (○, curve A) or 37 °C for 1 hour (●, curve B) with 2 nM-^{125}I-RBP and various concentrations of unlabelled native RBP. (*b*) The competition data from (*a*) were plotted in Scatchard form, and the continuous curves were calculated by using the best-fit parameter values. The parameter values are as follows: For curve A, $K_{D,1} = 3.0 \pm 2.7$ nM, $K_{D,2} = 95 \pm 35$ nM, $B_{max.,1} = 83 \pm 8.6$ and $B_{max.,2} = 8.0 \pm 4.8$; for curve B, $K_D = 79 \pm 5.5$ and $B_{max.} = 63 \pm 1.44$. $B_{max.}$ represents fmol of RBP bound/50 μg of membrane protein. The coefficient of multiple correlation values for both curves A and B is 0.99.

Competitive inhibition of ^{125}I-RBP binding by unlabelled RBP

Fig. 6(*a*) shows that the addition of increasing concentrations of native RBP resulted in a progressive decrease in the equilibrium binding (22 °C, 15 min) of ^{125}I-RBP to the membranes. Parallel competition studies were also performed using incubation conditions at 37 °C and 1 h in order to investigate the cause of decline in the equilibrium binding of ^{125}I-RBP under these conditions. The Scatchard plot (Fig. 6*b*) of the data obtained at 22 °C and 15 min was concave-upwards (curve A) suggesting some form of heterogeneity in the binding process. By using non-linear regression analysis, the calculated dissociation constants (K_Ds) for high- and low-affinity binding were $(3 \pm 2.7) \times 10^{-9}$ M and $(9.5 \pm 3.5) \times 10^{-8}$ M respectively. In contrast, Scatchard plots of the competition data, obtained under the incubation conditions of 37 °C and 1 h, were linear (curve B), suggesting a homogeneous binding reaction involving a single class of receptors and a single class of ligands. The estimated K_D of $(7.9 \pm 0.5) \times 10^{-8}$ M corresponds to low-affinity binding at 22 °C.

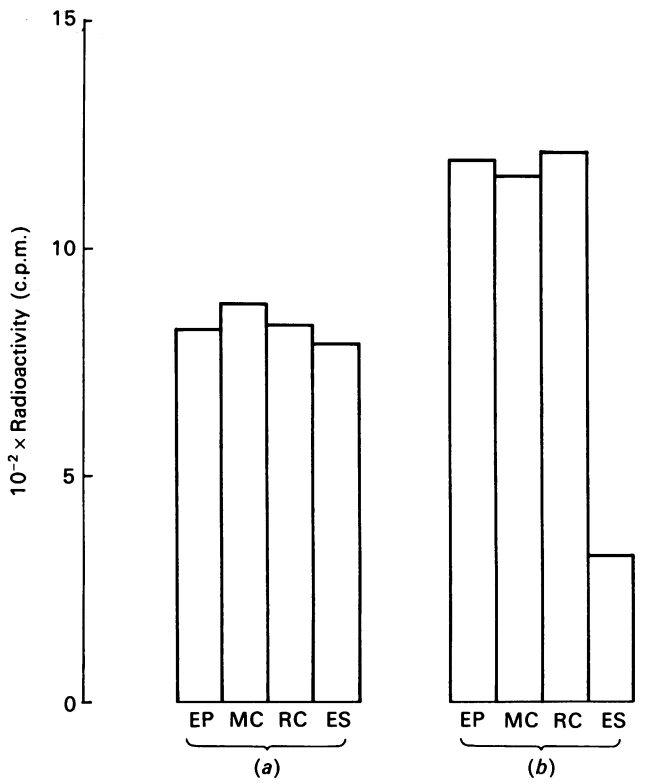

Fig. 7. Loss of high-affinity binding activity is due to RBP inactivation

Membranes (1 mg of protein/ml) were incubated at 37 °C for 60 min with ^{125}I-RBP (8 nM), then chilled and centrifuged. The supernatants (ES) and the pellet (EP) were separated. The binding characteristics of the supernatant ^{125}I-RBP (ES) and the pelleted membranes (EP) were examined by adding fresh membranes (1.0 mg of protein/ml) or fresh ^{125}I-RBP (8 nM) respectively and then monitoring the dissociation kinetics. Parallel incubations (controls) with membranes minus ^{125}I-RBP (MC) and with ^{125}I-RBP minus membranes (RC) were performed and their binding characteristics examined as described above. (*a*) Radioactivity (c.p.m.) released in the fast dissociation phase; (*b*) c.p.m. released in the slow dissociation phase.

RBP loses its high-affinity-binding property on interaction with the membranes

The kinetics of association and dissociation and Scatchard analysis of the binding process at 22 °C indicated heterogeneity of the binding sites or the ligands. It was also found that the equilibrium binding of RBP to the membranes declined on prolonged incubation, and

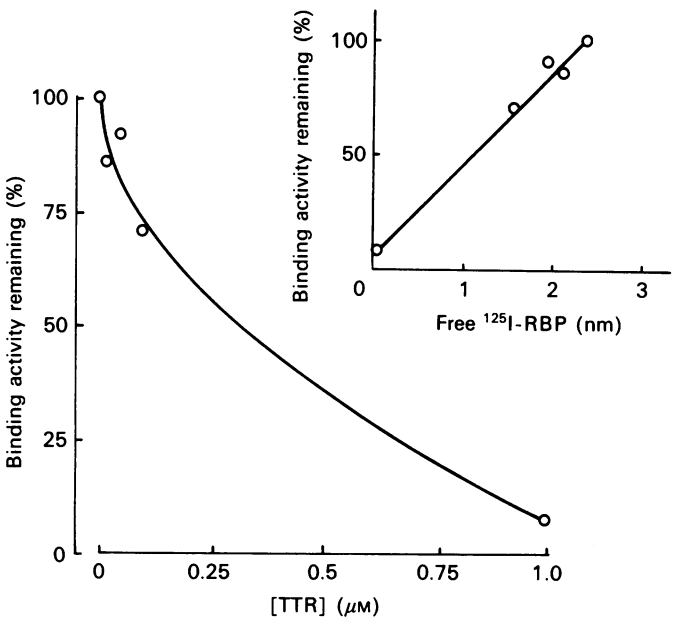

Fig. 8. Effect of TTR on the equilibrium binding of ^{125}I-RBP

Membranes (1.0 mg of protein/ml) were incubated at 22 °C for 10 min with 2.35 nM-^{125}I-RBP and various amounts of TTR. The inset shows a plot of calculated uncomplexed ^{125}I-RBP against percentage binding activity remaining.

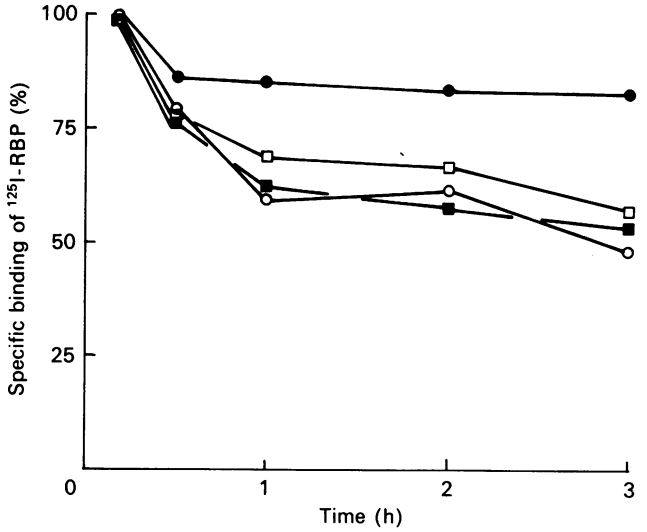

Fig. 9. Effect of TTR on the time course of ^{125}I-RBP binding

Membranes (0.97 mg of protein/ml) were incubated with ^{125}I-RBP (6 nM) at 22 °C in the absence (○) or in the presence of 0.1 μM-TTR (●), 10 μM-HSA (□) or 2 mM-PMSF (■). Samples were removed at various time intervals and assayed for the binding of ^{125}I-RBP as described in the Materials and methods section.

this was shown, by Scatchard analysis, to be due to the loss of high-affinity binding. But it was not clear whether this loss is associated with the inactivation of the receptor or of RBP. In order to resolve this, membranes were incubated with ^{125}I-RBP at a time point (60 min, 37 °C) after which further decline in the equilibrium binding was minimal, i.e. most of the high-affinity binding was lost. Membranes (EP) and unbound ^{125}I-RBP (ES) were then separated and their binding characteristics were separately examined, using fresh ^{125}I-RBP or membranes as appropriate, and monitoring the dissociation kinetics. Parallel control incubations with ^{125}I-RBP minus membranes (RBP control, RC) and membranes minus ^{125}I-RBP (membrane control, MC) were also analysed.

The results in Fig. 7 show that the binding properties of the membranes previously exposed to either ^{125}I-RBP (EP) or buffer alone (MC) were identical. On the other hand, a marked decrease in the slow-, but not the fast-, dissociating radioactivity was observed with ^{125}I-RBP (ES) which had previously been exposed to the membranes, as compared with ^{125}I-RBP which had been preincubated in buffer alone. The results therefore suggest the presence of at least two forms of RBP, with distinct receptor-binding kinetics, the slow-dissociating component corresponding to high-affinity binding.

Effect of TTR on binding of ^{125}I-RBP

Fig. 8 shows that human TTR inhibits the binding of ^{125}I-RBP in a concentration-dependent manner. When the data were plotted as percentage binding activity against the concentration of free ^{125}I-RBP, calculated by using a K_a value of 1×10^7 M^{-1} (Goodman, 1984) for TTR–RBP complex-formation, the resulting curve (Fig. 8, inset) was linear. Since only subsaturating concentrations of ^{125}I-RBP were used and the experiments were performed under pseudo-first-order conditions (5% of added ^{125}I-RBP was specifically bound), the linearity of the plot can be interpreted in terms of the interaction of free, rather than TTR-bound, RBP with the receptor. Alternatively, the inhibition could be due to a decrease in the affinity of RBP for the receptor when the former is complexed with TTR. The latter possibility cannot be ruled out from the data, since the curve does not pass through zero binding activity.

Fig. 9 shows that, in the presence of a 15-fold molar excess of TTR over RBP, the time-dependent decline in the equilibrium binding of RBP was lessened. In contrast with a 40% decline over 60 min in the equilibrium binding in the absence of TTR, the decline was less than 15% in the presence of TTR. This decline in binding was not prevented either by the inclusion of 2 mM-PMSF or by a 1000-fold molar excess of human serum albumin over RBP, suggesting that the effect of TTR was specific and not due to any proteolytic degradation of ^{125}I-RBP.

Effect of membrane modification on binding activity

Of the various reagents tested, iodoacetate, iodoacetamide, PMSF and NEM showed little or no effect on ^{125}I-RBP binding to the membranes (Table 1). PCMBS, on the other hand, was strongly inhibitory. The effect of PCMBS was only partially reversed by the addition of DTT. This is presumably because DTT itself caused some inhibition of binding. When PCMBS was reacted with DTT and the thiol groups of excess DTT were blocked by addition of NEM, all prior to the addition of membranes, no inhibition was observed. Since no free thiol groups are present in RBP (Goodman, 1984), and treatment of RBP with DTT before addition to the membrane did not cause any inhibition of the binding activity, inhibition by PCMBS and DTT is probably

Table 1. Effect of reagents on ^{125}I-RBP binding to placental microvilli

Membrane vesicles were incubated with the various reagents for 10 min at 37 °C, and binding activity for RBP was subsequently assayed. Reagents were added in the order shown. Excess DDISA was quenched with 50 mM-histidine (Barclay & Findlay, 1984) before binding activity was measured.

Addition	Concn. (mM)	Binding activity (%)
None	–	100
NEM	20	115±5.5
PCMBS	2	0
DTT	2	66±8.4
PCMBS+DTT	2,2	8±11
PCMBS+DTT+NEM	2,2,20	38.5±2
PCMBS+DTT+NEM*	2,2,20	105±7
Iodoacetamide	5	119±18
Iodoacetic acid	5	114±15
PMSF	2	109±3.5
DDISA	0.2	0
EDTA	10	99±1.0

* Membranes were added after ordered mixing and interaction of the reagents.

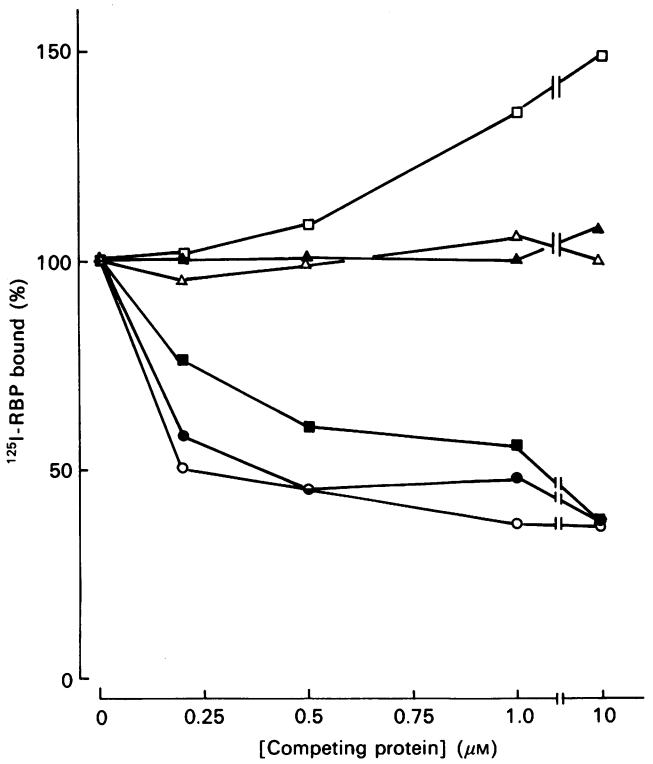

Fig. 10. Specificity of ^{125}I-RBP binding

Membrane vesicles (0.85 mg of protein/ml) were incubated with 2.3 nM-^{125}I-RBP for 15 min at 22 °C either alone (100% binding) or with the indicated concentrations of RBP (○), urinary RBP (●), serum albumin (□), TTR (■), transferrin (△) or bovine β-lactoglobulin (▲).

associated with thiol and disulphide groups on the membrane-bound receptor. Treatment of membranes with DDISA, which reacts with lysine, histidine, tyrosine and cysteine residues (Barclay & Findlay, 1984), abolished the specific binding of ^{125}I-RBP.

The RBP receptor was also trypsin- and heat-sensitive. Approx. 50% of the binding activity was lost when the membranes were treated with the proteinase (0.2 mg/ml) for 60 min at 37 °C. Almost 90% of the binding activity was lost when the membranes were heated at 70 °C for 10 min.

Specificity of RBP binding

The specificity of RBP binding was examined by monitoring the inhibitory potential of other binding proteins (Fig. 10). Only TTR and RBP (serum and urinary) decreased the amount of ^{125}I-RBP bound to membranes, indicating the strict specificity of the binding site. HSA, surprisingly, showed an increase in the binding activity of the RBP receptor (50% at 10 μM-HSA).

DISCUSSION

By using an oil-centrifugation assay, specific binding of ^{125}I-labelled RBP to human placental membrane vesicles could readily be demonstrated. The binding was reversible, and was time- and temperature-dependent. Both the association and dissociation kinetics of the binding process were biphasic. Although the overall binding reached equilibrium rapidly (15 min at 22 °C), prolonged incubation resulted in a subsequent decline (Fig. 2). Scatchard analysis of the equilibrium binding performed at 22 °C and 15 min, revealed high-[$K_D = (3\pm2.7)\times10^{-9}$ M] and low-[$K_D = (9.5\pm3.5)\times10^{-8}$ M] affinity binding of RBP to the membrane. However, in a parallel analysis, carried out using incubation conditions of 37 °C and 1 h, little of the high-affinity binding component could be detected (Fig. 6*b*). This was reflected in the loss of slow-dissociating ^{125}I-RBP binding from the membrane. Further, evidence was obtained to show that prolonged incubation of RBP with the membranes caused the loss of the high-affinity binding conformation of RBP, rather than any change in the membrane receptor (Fig. 7). Thus it would appear that the high-affinity form which associates instantaneously at 0 °C does not dissociate again at that temperature because it is not converted into the low-affinity form. The events at 22 °C are due to the slow-dissociating high-affinity form which does undergo conversion. Ligand variability of a similar kind has previously been identified as the cause of non-linear Scatchard plots for the binding of rat very-low-density lipoprotein to fibroblasts (Innerarity *et al.*, 1980).

It could not be established whether the high-affinity form of RBP, after delivering its retinol, loses its affinity completely for the receptor or is converted into the low-affinity form. The possibility of conversion into a low-affinity form could not be tested because the difference between K_D values is large, and only a small proportion of the total RBP as isolated existed in the high-affinity form. However, these observations provide experimental support for the proposition (Rask & Peterson, 1976) that RBP undergoes a marked loss in affinity for the receptor upon delivery of its bound retinol to the target cell. Retinol-uptake experiments [the following paper (Sivaprasadarao & Findlay, 1988)] suggest that retinol is

rapidly accumulated by placental membrane vesicles and that the apo-RBP remains outside. It seems possible, therefore, that the loss of high-affinity binding is the consequence of retinol delivery and that conformational changes in RBP are probably involved. Sandblom *et al.* (1986) have recently shown, for example, that removal of retinol from holo-RBP leads to closure of the entrance to the 'retinol barrel', and displacement of the *C*-terminal α-helix.

RBP circulates in the plasma as a 1:1 complex with TTR, presumably to prevent glomerular filtration of RBP, but the role of TTR in the cellular uptake of retinol is unclear. Rask & Peterson (1976) observed that TTR inhibited the uptake of RBP-bound retinol in monkey small-intestinal cells, and explained this observation in terms of a greater difficulty experienced by the receptor in removing retinol from RBP when complexed with TTR.

An alternative explanation might be a TTR-induced reduction in the availability of free RBP. Evidence for this was provided by the observation that ^{125}I-RBP binding to the receptor is inhibited by TTR in a manner that is directly proportional to the decrease in the concentration of free RBP (Fig. 8). Although the three-dimensional structure of the TTR–RBP complex is not yet available, indirect evidence from model-building (Newcomer *et al.*, 1984) and molecular-dynamics (Sandblom *et al.*, 1986) studies suggest that the protein structure lining the entrance to the retinol-binding pocket might constitute at least part of the interacting site with TTR. If this is true, then RBP freed of TTR may be a more convenient delivery vehicle for retinol. The observation that TTR prevents the decline in the equilibrium binding of ^{125}I-RBP with time (Fig. 9) can also be readily explained by the fact that TTR, by reducing the level of free RBP, decreases the rate of RBP inactivation by receptor interaction. The data thus suggest that retinol delivery to target cells might be controlled not only by the free RBP concentration in plasma, and the affinity and abundance of receptors on the target cells, but also by the ratio of TTR to RBP.

The inhibition of RBP binding by treatment of the membrane with trypsin, heat and various modification reagents strongly indicate that the RBP receptor is a protein. It seems to possess a rather specific site (presumably containing a thiol group) amenable to PCMBS, but not to NEM and other thiol-group-specific reagents, and perhaps (an) essential disulphide bridge(s) available to DTT. Interestingly, modification of a thiol group in the cardiac β-adrenergic receptor by organomercurials, but not by NEM, causes loss of ligand-binding activity (Strauss, 1984). Thiol groups which are reactive towards PCMBS, but not accessible to NEM and other thiol-group-specific reagents, have also been reported to be present in band 3 and the water-transport protein of erythrocyte membranes (Benga *et al.*, 1986). An alternative explanation for these results might be that it is the introduction of the bulky hydrophobic moiety of PCMBS rather than the modification of the thiol group that causes the loss of binding activity. However, since treatment of PCMBS with DTT followed by NEM, before the addition to membranes, did not affect binding activity, the presence of a PCMBS-sensitive thiol group is the favoured explanation. Treatment of membranes rather than ^{125}I-RBP with DTT markedly reduced binding, suggesting that maintenance of a disulphide bridge(s) in the receptor molecule may be essential for its activity.

The strict specificity of the RBP receptor was demonstrated by the inability of different serum proteins to inhibit RBP binding. Serum albumin, which under physiological conditions seems to carry retinoic acid, and is capable of binding retinol (Smith *et al.*, 1973), did not show any inhibitory effect. Indeed, it significantly increased the specific binding of ^{125}I-RBP to the receptor. It has also been reported that albumin increases the specific binding of concanavalin A to erythrocytes (Ketis & Grant, 1982), probably by a non-selective mechanism. Although it is not clear how albumin brings about such an effect with RBP, it may be of interest to note in this context that Chen *et al.* (1981) demonstrated that albumin greatly enhanced the enzymic transfer of retinol from rat liver cytosol retinyl ester–lipoprotein complex to apo-RBP. Significantly, β-lactoglobulin, which binds retinol and shows significant homology with human RBP in its amino acid composition and three-dimensional structure (Papiz *et al.*, 1986), had no effect on RBP binding.

In conclusion, the studies presented here demonstrate the presence of a highly specific protein receptor for RBP on human placental brush-border membranes. Studies on the role of this interaction on retinol uptake by the membrane are described in the following paper (Sivaprasadarao & Findlay, 1988).

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