Structure–function studies on human retinol-binding protein using site-directed mutagenesis

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Retinol-binding protein (RBP) transports vitamin A in the plasma. It consists of eight anti-parallel β -strands (A to H) that fold to form an orthogonal barrel. The loops connecting the strands A and B, C and D, and E and F form the entrance to the binding site in the barrel. The retinol molecule is found deep inside this barrel. Apart from its specific interaction with retinol, RBP is involved in two other molecular-recognition properties, that is it binds to transthyretin (TTR), another serum protein, and to a cell-surface receptor. Using site-directed mutagenesis, specific changes were made to the loop regions of human RBP and the resultant mutant proteins were tested for their ability to bind to retinol, to TTR and to the RBP receptor. While all the variants retained their ability to bind retinol, that in which residues 92 to 98 of the loop E-F were deleted completely lost its ability to interact with TTR, but retained some binding activity for the receptor. In contrast, the double mutant in which leucine residues at positions 63 and 64 of the loop C-D were changed to arginine and serine respectively partially retained its TTR-

binding ability, but completely lost its affinity for the RBP receptor. Mutation of Leu-35 of loop A-B to valine revealed no apparent effect on any of the binding activities of RBP. However, substitution of leucine for proline at position 35 markedly reduced the affinity of the protein for TTR, but showed no apparent change in its receptor-binding activity. These results demonstrate that RBP interacts with both TTR and the receptor via loops C-D and E-F. The binding sites, however, are overlapping rather than identical. RBP also appears to make an additional contact with TTR via its loop A-B. A further implication of these results is that RBP, when bound to TTR, cannot bind simultaneously to the receptor. This observation is consistent with our previously proposed mechanism for delivery of retinol to target tissues [Sivaprasadarao and Findlay (1988) *Biochem. J.* **255**, 571–579], according to which retinol delivery involves specific binding of RBP to the cell-surface receptor, an interaction that triggers release of retinol from RBP to the bound cell rather than internalization of retinol–RBP complex.

INTRODUCTION

Vitamin A is transported in the plasma as retinol bound to a specific carrier protein called retinol-binding protein (RBP). RBP, synthesized mainly in the liver, mobilizes hepatic stores of retinol to the plasma in a highly specific and regulated manner (for reviews see Goodman, 1984; Blomhoff et al., 1990). In the plasma, it circulates as a complex with another protein called transthyretin (TTR). Several studies (Heller, 1975; Rask and Peterson, 1976; Sivaprasadarao and Findlay, 1988a; Bavik et al., 1991) have shown that the delivery of retinol to vitamin A-requiring cells is mediated by specific receptors present in the plasma membrane of these cells, and that binding of RBP to these receptors triggers the transfer of retinol to the target cells. The resulting apo-RBP appears to lose its affinity for both the receptor and TTR, and, being small in size, is filtered in the kidneys and excreted in the urine. A different mechanism, however, may operate for retinol uptake by the liver, as the available evidence (Senoo et al., 1990) suggests that hepatocytes take up retinol by receptor-mediated endocytosis of the retinol–RBP complex.

Thus RBP, in spite of its smaller size, appears to be involved in three different molecular recognition events: it binds (1) to retinol, (2) to TTR and (3) to the cell-membrane receptor. The latter two interactions appear to be extremely specific. β -Lactoglobulin, in spite of its high degree of three-dimensional structural similarity and ability to bind retinol as well as RBP, does not interact with TTR (Papiz et al., 1986) or the RBP

receptor (Sivaprasadarao and Findlay, 1988a). These multiple interactions appear to have imposed evolutionary restraints on RBP evolution (Newcomer et al., 1984).

The structural basis for the retinol-binding function of RBP has become clear with the elucidation of its three-dimensional structure by X-ray crystallography (Newcomer et al., 1984; Cowan et al., 1990). The protein consists of eight antiparallel β -strands (A–H), which fold into two orthogonal sheets to form a flattened cone, calyx or barrel. The open end of the barrel is made up of loops which connect β -strands A–B, C–D, E–F and G–H. Retinol is bound within the barrel, with the hydroxyl group of the isoprene side-chain slightly exposed at the surface. A number of amino acid residues, mainly hydrophobic ones, line the binding pocket and make contact with the ligand.

In contrast, residues/regions of RBP involved in interaction with TTR and the membrane-bound receptor remain largely speculative. This is due to the absence of a crystal structure for the TTR–RBP complex and meaningful chemical modification data. In order to remedy this situation, we have used the approach of site-directed mutagenesis to help delineate the sites of interaction of RBP with its receptor and with TTR. The expression of RBP in *Escherichia coli* has been reported elsewhere (Sivaprasadarao and Findlay, 1993).

EXPERIMENTAL

Materials

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide

Abbreviations used: RBP, retinol-binding protein; rRBP, recombinant RBP; TTR, transthyretin; PMSF, phenylmethanesulphonyl fluoride; IPTG, isopropylthio- β -galactoside.

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kinase and Klenow fragment of DNA polymerase I were purchased from Boehringer-Mannheim. *Taq* DNA polymerase was from Perkin-Elmer/Cetus (Norwalk, CA, U.S.A.). Oligonucleotides used for mutagenesis were synthesized using an Applied Biosystems DNA synthesizer. The construction of the RBP expression vector, pOmp-RBP3 (Figure 1) has been reported previously (Sivaprasadarao and Findlay, 1993). *E. coli* XL-Blue (Stratagene) and BL21 (DE3) (Studier et al., 1986) were used for general cloning and expression respectively. All-*trans*-retinol, dimethyl sulphoxide, CNBr-activated Sepharose-CL-4B, and horseradish peroxidase-conjugated anti-(rabbit IgG) antiserum were purchased from Sigma Chemical Co. RBP and TTR were purified from human plasma as described previously (Sivaprasadarao and Findlay, 1988a). [³H]Retinol was obtained from ICN. Rabbit anti-RBP serum was a kind gift from Professor J. Glover, Liverpool University, Liverpool, U.K.

Construction of RBP mutants

Site-directed mutagenesis of RBP was performed using a modification of the PCR approach described by Landt et al. (1990). Figure 1 illustrates the steps involved in the procedure. The RBP expression vector, pOmp-RBP3, has the ribosome-binding site and the OmpA-RBP chimeric cDNA insert flanked by *Xba*I and *Hind*III restriction sites. On either side of these sites are vector sequences corresponding to the universal forward (*Xba*I side) and reverse (*Hind*III side) primers. Mutagenesis was performed in two PCR reactions using 5'-GTAAACGACGGCCAGTGAA-3', 5'-GGAAACAGCTATGACCATGAT-3' and mutagenic primers, incorporating the desired base change. The first PCR step was performed in a 100 µl mixture containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 1 mg/ml gelatin (*Taq* DNA polymerase buffer), 10 ng of the template DNA (pOmp-RBP3), 0.2 mM dNTPs, 100 pmol each of the reverse and mutagenic primers and 2.5 units of *Taq* DNA polymerase. Thirty cycles, each consisting of denaturation at 94 °C for 40 s, annealing at 50 °C for 1 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min, were performed in a Perkin-Elmer thermal cyclor. The resultant product was purified from agarose gels using DEAE (Na45) membranes (Schleicher and Schuell). The purified product was treated with 2 units of Klenow fragment of DNA polymerase I for 15 min at room temperature in a buffer containing 10 mM Tris/HCl, pH 8.0, 5 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol and 0.2 mM dNTPs in a 20 µl volume. The Klenow treatment which removes any nucleotides added to the 3' end of the PCR product was terminated by heating at 80 °C for 10 min. The second PCR step was performed using the first PCR product as one of the primers, the forward primer (100 pmol) as the second, and pOmp-RBP3 as template employing the conditions described above. The resultant new larger PCR product was purified from agarose gels, subjected to digestion with *Xba*I and *Hind*III, purified by extraction with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), precipitated with ethanol, and ligated into the *Xba*I and *Hind*III sites of pKS-Bluescript using T4 DNA ligase. *E. coli* XL-Blue cells were transformed with the ligation mixture and clones selected by their white colour on Luria-Bertani agar plates containing X-Gal, isopropylthio-β-galactoside (IPTG) and ampicillin. The method was highly efficient with over 90% of the resulting clones containing the desired mutation. Klenow treatment of the first PCR product was essential to avoid frame-shift mutations. All general cloning procedures used were as described in Sambrook et al. (1989).

The sequences of the primers used for site-directed

Figure 1 Site-directed mutagenesis of RBP cDNA

Details of construction are described in the Experimental section. Abbreviations: R, reverse primer; F, forward primer; M, mutagenic primer; RBS, ribosome-binding site; OmpA, OmpA signal sequence; φ 10, T7 promoter; Amp^r, ampicillin-resistance gene.

mutagenesis, and the changes (loops of RBP) made are shown below. The base changes are underlined.

5'-CGAGGGCCGCTTTCTGCAG-3';

L35P (loop A-B)

5'-CGAGGGCGTCTTTCTGCAG-3';

L35V (loop A-B)

5'-CGAGTCCGTCGTTCGAATAACTGG-3';

L63R/L64S (loop C-D)

Deletion of residues 92–98 of loop E-F of RBP was performed using the deletion-PCR protocol described by us previously (Sivaprasadarao and Findlay, 1993). Two oligonucleotides, one ending with the codon for residue 91 of RBP (anti-sense oligonucleotide, 5'-CCAGTACTTCATCTTGAA-3') and the other beginning with the codon for residue 99 of RBP (sense oligonucleotide, 5'-AAAGGAAATGATGACCAC-3') were used in a PCR to amplify all the sequence of pOmp-RBP3 except the nucleotides to be deleted (corresponding to residues 92–98 of RBP). A mixture of 1 nmol of each of the two oligonucleotide primers was phosphorylated using 20 units of T4 polynucleotide kinase for 1 h at 37 °C. PCR was performed in a 100 µl volume using 50 pmol of each phosphorylated primer and 10 ng of supercoiled pOmp-RBP3, *Taq* DNA polymerase buffer, 0.2 mM dNTPs and 5 units of *Taq* DNA polymerase. Thirty PCR cycles, each consisting of 40 s at 94 °C (denaturation), 30 s at 46 °C (annealing) and 10 min at 72 °C (extension), with a final extension for 10 min at 72 °C, were performed. The resulting product was purified from agarose gels, subjected to Klenow treatment (see above), purified using the phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) extraction and ethanol precipitation, and self-ligated using T4 DNA ligase. *E. coli* XL-Blue cells were transformed with the ligation mixture and selected on ampicillin plates. It is important to use a supercoiled form of the template plasmid DNA in this deletion protocol to maximize the mutant yield. Several clones were sequenced using the dideoxy method of Sanger et al. (1977) and those with the desired mutations, but devoid of any PCR-borne errors, were used for expression studies.

Expression and purification of mutant RBPs

Plasmids encoding the wild-type or mutant forms of RBP were transformed into *E. coli* strain BL21 (DE3) and grown in a 2 litre culture flask at 37 °C and 200 rev./min in 600 ml of M9 casamino acid medium (Sambrook et al., 1989) containing 100 µg/ml of ampicillin. When the absorbance value (A_{600}) reached 0.6–0.7 expression was induced by the addition of IPTG to 1 mM. After growth for a further 4 h at 30 °C and 200 rev./min the cells were harvested by centrifugation. The periplasm was extracted from the pelleted cells as described before (Sivaprasadarao and Findlay, 1993). The expression of mutant RBPs was examined by SDS/PAGE, followed by Western blotting. After incubation with retinol, the crude periplasmic extracts were subjected to purification on TTR-coupled Sepharose CL-4B as described by Vahlquist et al. (1971). The RBP content of the periplasmic extracts, column flow-throughs and the eluted fractions was estimated by r.i.a. as described by Blaner (1990), and spectrophotometry where purification of the mutant protein was possible. Only two of the four mutants, L35P and L35V, could be purified using the TTR-affinity resin.

Assay of retinol binding to mutant RBPs

Binding of retinol to mutant RBPs present in the crude periplasmic extracts was tested using [³H]retinol in a gel-filtration assay system. Extracts containing RBPs were incubated in the dark for 2 h at 37 °C with [³H]retinol in the absence (total) or in the presence (non-specific) of a 1000-fold excess of unlabelled retinol. The extracts were then applied to Sephadex G-100 columns (60 cm × 1.8 cm) calibrated with molecular-mass markers, and serum RBP. The proteins were eluted with 10 mM Tris/HCl, pH 7.5, and the fractions assayed for radioactivity. Control incubations using extracts obtained from cells containing the parent plasmid, pKS-Bluescript, were also carried out. A radioactive peak, corresponding to the elution position of serum RBP, present in the 'total' incubations but absent in the 'non-specific' incubations, was taken as an indication of retinol binding to the mutant proteins.

Assay of TTR-binding activity of mutant RBPs

Freeze-dried periplasms, derived from 1 litre of appropriate *E. coli* cultures expressing the mutant proteins, were dissolved in 10 ml of equilibration buffer [50 mM Tris/HCl/0.2 M NaCl, pH 7.4/0.2 mM phenylmethanesulphonyl fluoride (PMSF)] and incubated in the dark with 0.1 mM retinol (added from a stock in dimethyl sulphoxide) for 2 h at 37 °C. The mixture was centrifuged at 10000 *g* for 10 min, and the clear supernatant was circulated through 5 ml of TTR-Sepharose CL-4B in a column overnight. The flow-through was retained, and after washing the column with six bed volumes of equilibration buffer, the bound protein was eluted with distilled water, the pH of which was adjusted to 8.0 with ammonia. The concentration of RBP in the crude periplasms, flow-throughs and eluate was estimated using r.i.a. The percentage of loaded RBP bound by the TTR-affinity resin was calculated from these estimates. The assays were done twice in duplicate.

Assay of receptor-binding activity of mutant RBPs

The periplasmic extracts from appropriate *E. coli* cultures (1 litre) expressing the mutant forms of RBP were concentrated, dialysed against water, freeze-dried and dissolved in 1 ml of 20 mM sodium phosphate/0.15 M NaCl, pH 7.4. After removing the insoluble material by centrifugation, the clear supernatant was used to purify the expressed wild-type recombinant RBP (rRBP) and L35V mutant. Binding of mutant RBPs to the placental membrane receptor was performed using ¹²⁵I-labelled rRBP in a competition assay (Sivaprasadarao and Findlay, 1988a). The binding activity of other mutants was examined using crude preparations. Periplasm, obtained from *E. coli* not expressing RBP, served as a control. All assays were performed in triplicate.

RESULTS AND DISCUSSION

Using site-directed mutagenesis, we have attempted to identify residues/regions of RBP involved in its interaction with TTR and with the cell-surface receptor. In choosing the areas for mutagenesis, we examined the published three-dimensional structure of RBP (Newcomer et al., 1984; Cowan et al., 1990) and its relationship with other members of the RBP superfamily (Papiz et al., 1986; Cowan et al., 1990). We also considered the information available from chemical modification studies (Horwitz and Heller, 1974) and molecular dynamic simulations (Aqvist et al., 1986). Residues of the surface-exposed loops connecting strands A-B, C-D and E-F appeared to be likely

candidates in one or both of the interactions for several reasons. First, changing these residues does not alter the β -core structure of the protein, which is essential for retinol binding, which in turn seems to be essential for interaction with TTR and the receptor. Secondly, these loops form the entrance/exit site for retinol and therefore are the regions more likely to be involved in binding to the receptor if the mechanism for retinol delivery proposed by us (Sivaprasadarao and Findlay, 1988b) proves to be correct. Thirdly, molecular dynamic simulations indicated that the entrance loops of RBP are likely to be involved in binding to TTR (Aqvist et al., 1986). And finally, multiple alignment of the primary (Cowan et al., 1990) and tertiary structures of RBP (Papiz et al., 1986; Cowan et al., 1990) with other members of the lipocalin superfamily indicates that these loops represent highly variable regions, both in size, sequence and spatial position.

Leu-35 in loop A-B was mutated to proline (L35P) in order to introduce a slight change to the already irregular structure of this region without altering the polar properties of this position. The same residue was also changed to valine, a conservative substitution not expected to alter the structure or characteristics of the region. In contrast, leucine residues at positions 63 and 64 of loop C-D were substituted by arginine and serine respectively, which because of their respective charged and polar characteristics should substantially change the properties of this region. As several residues of loop E-F appear to be available for interaction with TTR and/or the receptor, we deleted the entire loop (des[92–98]). A PCR-based procedure, illustrated in Figure 1, was used to introduce single base changes. Deletion of residues 92–98 of the loop E-F was carried out using deletion PCR (Sivaprasadarao and Findlay, 1993). The levels of expression of the various mutants varied; those of mutants L35P and L35V, and the double mutant L63R/L64S, were of the same order as the wild-type RBP (200–300 $\mu\text{g/l}$ of culture), while the yield of the deletion mutant, des[92–98], was much higher, about 2 mg/l.

Binding of RBP variants to retinol

Binding of retinol to the mutant proteins was examined by spectrophotometry and/or [^3H]retinol binding in a gel-filtration assay. Of the four mutant proteins, L35P and L35V could be purified by TTR-affinity chromatography from retinol-treated periplasms. These two purified mutant proteins showed an $A_{330/280}$ ratio of 1.0, which is characteristic of holo-RBP. Binding of retinol to the double mutant, L63R/L64S, and the deletion mutant, des[92–98], was examined by incubating the periplasm containing the expressed mutant protein with [^3H]retinol and fractionating them on a calibrated Sephadex G-100 column. A radioactive peak, displaceable by excess unlabelled retinol, corresponding to serum RBP peak was observed with all the mutant RBPs examined. Thus, none of the mutational changes to RBP appears to have altered the ability of RBP to bind retinol. Although the crystal structure suggests that Leu-35 of loop A-B, Leu-63 of loop C-D, and Phe-96, Leu-97 and Gln-98 of loop E-F of RBP appear to be close to the terminal part of the isoprene side-chain of retinol (Newcomer et al., 1984; Cowan et al., 1990), alteration or deletion of these residues seems to have little effect on ligand binding. This is probably due to the fact that a large number of other residues lining the inside of the binding pocket make a more substantial contribution to the binding of retinol.

Binding of RBP variants to TTR

The binding of the RBP mutants to TTR was examined by testing their ability to be retained by a TTR-affinity resin. The

Table 1 Binding of mutant forms of RBP to TTR

RBP variant	Percentage bound
Wild-type	100
L35V	88 ± 15
L35P	12 ± 1.2
L63R/L64S	2.2 ± 1.0
Des[92–98]	0

periplasmic extracts containing the expressed wild-type and mutant proteins were incubated with retinol before circulation through the affinity column. After washing with the starting buffer, the bound proteins were eluted with distilled water adjusted to pH 8.0 with ammonia. The amounts of RBP present in the crude periplasmic extracts, column flow-throughs and eluted fractions were quantified by r.i.a. The results presented in Table 1 show that while the TTR-binding ability of the mutant L35V did not change significantly from that of the wild-type RBP, the L35P mutant showed a marked decrease in binding. The double mutant, L63R/L64S, as well as the deletion mutant, des[92–98], also completely lost their TTR-binding abilities. As all the mutants bound retinol, the results are not due to their being in the apo-form. At the end of these experiments, the resin was tested using pure serum RBP and no significant change was found in its RBP-binding capacity, thereby eliminating any possible inactivation/degradation of the resin-bound TTR during chromatography of the bacterial extracts.

Several approaches, both biochemical and biophysical, have been employed to identify the regions of RBP involved in binding to TTR. Horwitz and Heller (1974) showed that treatment of RBP with 2-hydroxy-5-nitrobenzyl bromide modifies two out of the four tryptophan residues of RBP and that this modification inhibits complex formation with TTR, without affecting retinol binding. Cowan et al. (1990) noticed in their crystal structure of RBP that Trp-24 and Trp-105 were buried in the molecule, whereas Trp-67 (in loop C-D) and Trp-91 (in loop E-F) were exposed, and suggested that the latter two were probably amenable to chemical modification. They suggested that residues in the loops C-D and E-F were more likely to be close to or part of the TTR-binding site. Cowan et al. (1990) also noticed that the loop region around Asn-65 of loop C-D was poorly defined in their maps and suggested that the conformational flexibility of the C-D loop may be stabilized by the binding of TTR. Recently, Aqvist and Tapia (1992) published a molecular model for the RBP–TTR complex using interactive rigid-body computer graphics surface docking. The authors suggested that loops C-D (around Asn-65) and E-F (around Ser-95) of RBP are buried in a Y-shaped canyon formed by TTR. Our present results provide direct experimental support for these predictions. Most of the mutations that we have made to RBP involved changes from hydrophobic to charged and hydrophilic residues or removal of several hydrophobic residues. The results thus indicate the hydrophobic nature of the binding forces between RBP and TTR and help explain the observed dissociation of the RBP–TTR complex at low ionic strength (Vahlquist et al., 1971). It is interesting to note that the loop E-F is larger in RBP than in any other members of the RBP superfamily, including purpurin (Berman et al., 1987) the closest analogue of RBP, and that no other member of this family appears to be able to bind to TTR. The TTR-interacting specificity thus appears to be mainly associated with this loop.

Table 2 Competitive inhibition of ^{125}I -RBP binding to placental membranes by RBP variants

1.2 nM ^{125}I -labelled rRBP (82 000 c.p.m.) was incubated with placental membrane vesicles (1.3 mg/ml) in the absence and the presence of RBP variants at the indicated concentrations at 37 °C for 10 min. Membrane-bound ^{125}I -RBP was determined as described in the Experimental section.

RBP variant	rRBP concn. (μM)	^{125}I -RBP bound (c.p.m.)
None		17 756 $\pm$ 810
Wild type	0.5	7608 $\pm$ 875
L35V	0.5	7560 $\pm$ 239
L35P	0.5	7773 $\pm$ 551
L63R/L64S	1.0	18 094 $\pm$ 983
Des[92–98]	0.5	16 525 $\pm$ 1238
Des[92–98]	2.0	14 858 $\pm$ 302

Figure 2 Receptor- and TTR-binding determinants identified for human RBP by site-directed mutagenesis

The three-dimensional structure of human RBP was retrieved from the Protein Data Bank, Brookhaven. Residues of RBP which have been mutated or deleted are labelled. Residues Leu-35, Leu-63 and Leu-64 and the loop connecting the strands E and F (residues 92–98) appear to be involved in TTR binding (listed in the increasing order of their binding strengths). Leu-63 and Leu-64 of loop C-D and loop E-F (residues 92–98) are involved in interaction with the receptor, the former making stronger contact than the latter.

Receptor-binding activity of mutants

The receptor-binding activities of the RBP mutants were determined in a competitive assay using ^{125}I -labelled RBP and human placental membrane vesicles. We have previously demonstrated that the receptor-binding activity of the bacterially produced RBP is higher than that of the plasma-derived RBP (Sivaprasadarao and Findlay, 1993) and therefore all binding activities were normalized to that activity. Table 2 shows that mutation of Leu-35 to Pro or Val had no apparent effect on the

binding of RBP to its receptor. The L63R/L64S mutant, on the other hand, completely lost its receptor-binding activity. Deletion of residues 92–98, which are largely hydrophobic, of the loop E-F significantly decreased the receptor-binding activity. Thus loop C-D appears to make a stronger contact with the receptor than the loop E-F. The results tend to suggest that the binding of RBP to TTR may involve hydrophobic interactions. This is consistent with our observation that RBP–receptor complex dissociates rapidly in low ionic strength buffer at physiological pH (Sivaprasadarao et al., 1994). Thus the formation of complexes of RBP with TTR and the receptor appears to involve several common hydrophobic amino acid residues and consequently, both the complexes exhibit similar physicochemical behaviour in that both are unstable in low ionic strength buffers.

The mechanism of uptake of retinol from plasma RBP by vitamin A-requiring cells has been somewhat controversial. Three different possibilities, with varying degrees of evidence, have been put forward. A diffusion mechanism, according to which retinol simply diffuses from RBP into the lipid bilayer of the cell, has been proposed by Fex and Johannesson (1988) and Noy and Xu (1990). However, the grossly unequal distribution of retinol among various tissues of the body and its absence from erythrocytes and several other potentially retinol-binding blood components, including albumin and lipoproteins, remains to be convincingly explained. Others (Heller, 1975; Rask and Peterson, 1976; Sivaprasadarao and Findlay, 1988a; Bavik et al., 1991) suggest involvement of a receptor. The receptor-mediated transport, being extremely specific, offers a distinct physiological advantage to the organism by not only directing the delivery of retinol to tissues that require vitamin A for function but by also preventing cellular damage by free retinol. The experimental data obtained with pigment epithelial cells (Chen and Heller, 1977) and placental membranes (Sivaprasadarao and Findlay, 1988b) strongly suggest that RBP binding to the receptor results in the transfer of retinol to the bound cell, with the resultant apo-RBP remaining outside. With hepatocytes (Senoo et al., 1990), and more recently with a variant F9 embryonal carcinoma cell line (Matarese and Lodish, 1993), evidence for receptor-mediated endocytosis of RBP has been presented.

If the receptor-mediated transfer mechanism were correct, the obvious escape route for retinol from RBP is the open end of the β -barrel, which is surrounded by the loops A-B, C-D and E-F. The other end, being closed by several phenylalanine residues, does not appear to provide an exit route. The crystal structure of RBP shows that Leu-35 of loop A-B, Val-61 and Leu-63 of loop C-D and Phe-96, Leu-97 and Gln-98 of loop E-F make contact with the terminal part of the isoprene side-chain of retinol. Cowan et al. (1990) suggested that residues Gln-98 and Leu-35 could control the entrance/exit to the barrel, and changing their side-chain conformation may open up the binding site to the solvent. Recently, Zanotti and co-workers published the crystal structures of apo-RBP isolated from human plasma (Zanotti et al., 1993a) and bovine plasma (Zanotti et al., 1993b). Superposition of the three-dimensional structures of apo- and holo-forms of human RBP revealed significant conformational differences around residue Leu-35 (i.e. residues 34–37). No significant differences were apparent in the rest of the molecule. However, our present mutagenic study shows that Leu-35 is unlikely to be directly involved in binding to the receptor, but the apparently flexible loop, C-D, probably makes a strong hydrophobic contact with the receptor, aided by the residues of loop E-F. It seems possible that apo-RBP crystallized by Zanotti et al. (1993a,b) is not the physiological form, but is generated during the storage of serum. Loss of retinol from RBP during storage, as well as heterogeneity of RBP, have been reported by several

groups (Raz et al., 1970; Peterson, 1971). It thus appears that the conformation of the physiological form of RBP is likely to be distinct from that of the apo-RBP generated *in vitro*. Information on the three-dimensional structure of the former is crucial to delineate the molecular details of the transfer process.

Taking into consideration the available three-dimensional structure of RBP and the present mutagenic study, one could envisage the following mechanism for the cellular uptake of retinol. The binding of RBP to the receptor by its surface loops probably induces some conformational changes that promote the release of retinol to the bound cell by disrupting the bonds that hold retinol in the binding pocket. This disruption probably begins at the entrance/exit region of RBP and propagates down the barrel leading to the opening of the pocket, and the consequent transfer of retinol to the bound cell. This proposal is hard to verify experimentally, but a combination of structural, biochemical and molecular biological approaches is likely to provide some insights into the molecular details of the transfer process. Recent reports (Bavik et al., 1992; Sivaprasadarao et al., 1993) of the purification of the receptor offer some promise towards achieving this goal. More recently, Bavik et al. (1993) reported the cloning of an RBP receptor from bovine pigment epithelial cells by expression cloning using a monoclonal antibody raised to the putative receptor as a probe. The clone encodes a 63 kDa protein but one which does not appear to possess either the usual structural features of a membrane receptor/transport protein or a signal sequence characteristic of an extracellular protein. RBP-receptor-binding activity of this clone, expressed in mammalian cells, has yet to be demonstrated.

Another interesting outcome of the present mutagenic study (Figure 2) is that it clearly demonstrates that the TTR- and receptor-binding sites on RBP are overlapping. Interaction with both involves loops C-D and E-F. However, RBP appears to bind TTR more strongly with loop E-F than with loop C-D, while the converse is true for the receptor. The implication of this result is that RBP is most unlikely to bind to its receptor when complexed to TTR. We have previously demonstrated (Sivaprasadarao and Findlay, 1988a) that TTR inhibits ^{125}I -RBP binding to the receptor. Moreover, it is the rate of uptake, but not the equilibrium uptake, of [^3H]retinol from its RBP complex by the target cell membranes (Sivaprasadarao and Findlay, 1988b) that is reduced when RBP is complexed to TTR. These studies suggested that it is free, rather than the TTR-complexed RBP, that binds to the receptor. Others (Rask and Peterson, 1976; Bavik et al., 1991) are of the opinion, however, that the slow uptake of retinol from the RBP-TTR complex compared with free RBP is due to the difficulty in removing retinol from the TTR-complexed RBP. The present mutagenic study clearly demonstrates that binding of TTR occludes the receptor-binding sites of RBP and prevents its interaction with the receptor.

RBP is the first member of the rapidly expanding lipocalin superfamily on which a mutagenic study has been carried out. As the family is characterized by a highly conserved β -core structure, it is possible to design and perform interesting experiments, such as grafting the loops of RBP into equivalent positions in other members of the family (e.g. β -lactoglobulin), and to observe

whether the RBP receptor and/or TTR-binding functions can be conferred to these members, and whether ligands associated with these proteins can be directed to cells via the RBP receptor. Similar studies with other members of the family which may possess receptors raise the possibility of targeted drug delivery, particularly as the binding site itself may be engineered to provide defined specificity. Such studies will help further our understanding of the structure-function relationships of the lipocalin superfamily.

The work reported here is supported by a grant from the Agricultural and Food Research Council, U.K. to Lindsay Sawyer (Biochemistry Department, University of Edinburgh, Edinburgh, U.K., and J.B.C.F.).

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